T Regulatory Cells in Allergic Disease

Dissertation

zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der
Universität Zürich
von

Johan Verhagen

aus
den Niederlanden

Promotionskomitee

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Zürich, 2006

Publications:

1. Absence of T-regulatory cell expression and function in atopic dermatitis skin. J Allergy Clin Immunol., 2006. 117:176-83

2. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med., 2004. 199: 1567-1575

3. A second step of chemotaxis after transendothelial migration: keratinocytes undergoing apoptosis release IFN-γ-inducible protein 10, monokine induced by IFN-γ, and IFN-γ-inducible α-chemoattractant for T cell chemotaxis toward epidermis in atopic dermatitis. J Immunol., 2003. 171: 1078-1084

Contents

1. Acknowledgements

After spending four years in a place it is always hard to name everybody who has contributed to making this time successful and memorable. First of all, I would like to thank Prof. Dr. K. Blaser for giving me the opportunity to work as a PhD student at the Swiss institute of allergy and asthma research (SIAF), in Davos. Secondly, I thank Prof. Dr. Cezmi A. Akdis for taking me up in his group, helping me with my projects and giving me the opportunity to publish several scientific papers and ultimately this thesis. Prof. Dr. Roland H. Wenger kindly agreed to become the responsible faculty member at the science faculty (MNF) of the University of Zurich, and therefore played an important part in the completion of this work.

Next, I would like to thank Dr. Sven Klunker and PD Dr. Mübeccel Akdis for the collaborations, which have led to two of the publications in this thesis. Both also highly contributed to the pleasant working atmosphere within the group. For this, their collaboration in shared projects, and general friendship, I thank all past and present members of the “upstairs” group, in particular buddy and room mate Dr. Alison Taylor, Judith “Jubi” Zumkehr, Flurina “Fluurli” Meiler and Maya Zimmermann. Furthermore, I would like to thank all other past and present members of the institute, who have been helpful colleagues and/or good friends. Many research groups from outside the institute have also contributed to this work as part of international cooperation. Please check the articles in the results section for their names. Finally, the people of Davos have made it a great place to live and work.

This work was supported by the Swiss National Science Foundation grants No. 31-105865 and 32-100266 and the Global Allergy and Asthma European Network (GA^2LEN).

2. Abbreviations

AD:	atopic dermatitis
AE:	atopic eczema
AICD:	activation-induced cell death
APC:	antigen-presenting cell
CLA:	cutaneous lymphocyte-associated antigen
CTL:	cytotoxic T lymphocyte
CTLA-4:	cytotoxic T lymphocyte antigen-4
DC:	dendritic cell
EAD:	extrinsic (allergic) type atopic dermatitis
ECM:	extracellular matrix
EDC:	epidermal dendritic cell
FasL:	Fas ligand
GITR	glucocorticoid-induced tumour necrosis factor receptor
GM-CSF:	granulocyte macrophage-colony stimulating factor
GVHD:	graft-versus-host disease
HDM:	house dust mite
IAD:	intrinsic (non-allergic) type atopic dermatitis
ICOS:	inducible costimulator
IFN-γ:	interferon-γ
Ig:	immunoglobulin
IL:	interleukin
IP-10	interferon-γ-inducible protein-10
IPEX:	immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome
iTac	interferon-γ-inducible-α-chemoattractant
KC:	keratinocyte
LC:	Langerhans' cell
mAb:	monoclonal antibody
MBP:	myelin basic protein

Mig	monokine induced by γ-interferon
NK:	natural killer
PBMC:	peripheral blood mononuclear cells
PD-1:	programmed death-1
PI3K:	phosphatidylinositol 3-kinase
PIT:	peptide immunotherapy
SIT:	specific immunotherapy
TCR:	T cell receptor
TGF-β:	transforming growth factor-β
Th:	T helper
TLR:	Toll-like receptor
TNF-α:	tumour necrosis factor-α
Tr1:	type 1 regulatory T cell
Treg:	T regulatory
VCAM-1:	vascular cell adhesion molecule-1
XLAAD:	X-linked autoimmunity–allergic dysregulation syndrome

3. Summary

Allergic disease is controlled, mediated and in some cases executed by T cells. Upon the recognition of allergen, T helper 2 (Th2) cells induce the secretion of immunoglobulin (Ig)E by B cells, leading to mast cell activation and the release of inflammatory mediators, such as histamine and leukotrienes. This forms the basis of the common allergic type 1 hypersensitivity response. Th1 cells play an important role in the chronicity of allergic inflammation and are responsible for the major tissue injury event in atopic dermatitis; the induction of apoptosis in epidermal keratinocytes. T regulatory cells play an important role in the control of inflammation and, as shown in this thesis, are key regulators of the healthy immune response to allergens.

Atopic dermatitis is a chronic inflammatory skin disease characterised by Th1 cell-induced keratinocyte apoptosis. This key tissue injury event is mediated through interferon-γ (IFN-γ) and Fas, expressed by Th0/Th1 cells in the dermal and epidermal layers of the skin. We showed that keratinocytes undergoing apoptosis actively aggravate the inflammation. Keratinocyte stimulation with IFN-γ induces the upregulation of chemokines such as interferon-γ-inducible protein-10 (IP-10), monokine induced by γ-interferon (Mig) and interferon-γ-inducible α chemoattractant (iTac). These chemokines attract T cells bearing the specific receptor CXCR3. This facilitates the further infiltration of T cells into the skin and towards the epidermis, leading to the formation of eczematous lesions.

The balance between Th2 and Th1 cells has long been considered decisive for an allergic or healthy immune response to allergens. Although Th1 cells suppress the proliferation of Th2 cells and can therefore prevent a type 1 hypersensitivity response, the induction of a Th1 response could lead to a type IV hypersensitivity with equally undesired effects. We have shown that it is rather the balance between allergen-specific IL-10-secreting T regulatory type 1 (Tr1) cells and Th2 cells that decides the outcome of an allergen encounter. Healthy

individuals have a higher prevalence of allergen-specific Tr1 cells, while allergic individuals show a majority of allergen-specific Th2 cells. The Tr1 cells are highly effective suppressors of T cell activation and utilise a combination of the secreted suppressive cytokines IL-10 and TGF-β, but also rely on the cell surface receptors CTLA-4 and PD-1. Since we have further shown that these Tr1 cells can be expanded *in vitro* with a range of cytokines (IL-2, IL-4, IL-7 and IL-15), the induction of allergen-specific Tr1 cells forms a potential treatment modality in allergen-specific immunotherapy (SIT).

Although we and others have shown that both Tr1 cells and $CD4^+CD25^+FoxP3^+$ Treg cells are capable of suppressing T cell activation *in vitro*, as well as that the number of Treg cells in the blood positively correlates with reduced allergic and autoimmune disease, little is known about the presence and role of Treg cells in the tissues. We examined lesional skin biopsies of patients suffering from atopic dermatitis or a number of other conditions and found that although there are numerous IL-10- and TGF-β-secreting Tr1 cells in the dermis and epidermis, there are no $CD4^+CD25^+FoxP3^+$ Treg cells.

Subsequently, we have performed co-culture experiments of both types of Treg cells, in combination with keratinocytes and activated Th1 cells, in order to determine whether these cells, which are highly capable of suppressing T cell activation, are capable of suppressing the major tissue injury event in atopic dermatitis, Th1 cell-induced keratinocyte apoptosis. Experiments of keratinocytes combined with T cells or only cytokines showed that both types of Treg cells are incapable of suppressing the effector functions of pre-activated T cells.

In conclusion, we have demonstrated that Treg cells play an important role in the maintenance of T cell unresponsiveness to allergens. The induction of allergen-specific Treg cells by allergen-SIT could lead to an immunological state where an allergic response upon allergen encounter is no longer generated. However, when pre-activated T cell are actively drawn to the tissue under the influence of a

chemokine network, Treg cells cannot suppress the destructive effects of apoptosis-inducing effector T cells.

4. Zusammenfassung

Allergische Erkrankungen werden von T Zellen kontrolliert, vermittelt und in einigen Fällen auch verursacht. Nach dem Erkennen eines Allergens induzieren T Helfer 2 Zellen (Th2) die Freisetzung von Immunglobulin (Ig) E durch B-Zellen. Das wiederum führt zur Aktivierung von Mastzellen und der Freisetzung entzündlicher Mediatoren, wie Histamin und Leukotrienen. Dieses bildet die Grundlage der allergischen Hypersensitivitäts-Antwort 1. Th1 Zellen spielen eine wichtige Rolle in der chronischen allergischen Entzündung. Sie sind deshalb auch hauptsächlich für die Beschädigung des Gewebes in der atopischen Dermatitis verantwortlich, die vor allem durch die Induktion von Apoptose in den epidermalen Keratinozyten zu Stande kommt. Regulatorische T Zellen nehmen eine wichtige Position bei der Kontrolle der Entzündung ein und spielen eine Schlüsselrolle als Regulatoren einer gesunden Immunantwort auf Allergene.

Atopische Dermatitis ist eine chronisch-entzündliche Hauterkrankung, die durch Th1 induzierte Keratinozyten-Apoptosis charakterisiert ist. Die Beschädigung von Gewebe wird hauptsächlich durch IFN-γ und Fas vermittelt, welche von Th0/1 Zellen in der Dermis und Epidermis gebildet werden. Wir zeigten, dass Keratinozyten, die in Apoptose übergehen aktiv die Entzündung verschlimmern. Die Stimulierung von Keratinozyten durch IFN-γ induziert die Bildung von Chemokinen, wie IP-10 (interferon-γ-inducible protein), Mig (monokine induced by interferon-γ) und iTac (interferon-γ-inducible-α-chemoattractant). Diese Chemokine locken T Zellen mit dem spezifischen Rezeptor CXCR3 an. Das erleichtert das weitere Eindringen von T Zellen in die Haut und bis in die Epidermis und führt zur Entstehung ekzematöser Läsionen.

Das Gleichgewicht zwischen Th2 und Th1 Zellen wurde lange Zeit für entscheidend gehalten, ob es zu einer allergischen oder einer gesunden Immunantwort auf ein Allergen kommt. Obgleich Th1 Zellen die Proliferation von Th2 Zellen unterdrücken, und damit die Typ-1-Hypersensitivitäts-Antwort

verhindern, könnte die Induktion einer Th1 Antwort zu einer Typ IV Hypersensitivität mit genauso unerwünschten Folgen führen. Wir haben gezeigt, dass stattdessen das Gleichgewicht zwischen Allergen-spezifischen IL-10-absondernden regulatorischen T Zellen vom Typ 1 und Th2 Zellen entscheidend für das Ergebnis nach dem Zusammentreffen mit einem Allergen ist. Gesunde Personen haben eine höhere Prävalenz von allergen-spezifischen Tr1 Zellen, während allergische Personen vor allem allergen-spezifische Th2 Zellen aufweisen. Tr1 Zellen sind hoch-wirksame Suppressoren der T Zellaktivierung. Sie verwenden eine Kombination von freigesetzten Suppressor-Zytokinen IL-10 und TGF-β, aber auch die Zelloberflächenrezeptoren CTLA-4 und PD-1 zur Unterdrückung. Wie wir schon vorher gezeigt haben, können diese Tr1-Zellen *in vitro* mit Hilfe verschiedener Zytokine (IL-2, IL-4, IL-7 und IL-15) expandiert werden. Die Induktion von allergen-spezifischen Tr1 Zellen stellt somit eine Behandlungsmöglichkeit in der allergen-spezifischen Immuntherapie (SIT) dar.

Obwohl wir und auch andere gezeigt haben, dass Tr1 und $CD4^+CD25^+FoxP3^+$ Treg Zellen in der Lage sind T Zellenaktivierung *in vitro* zu unterdrücken und weiterhin die Anzahl der Treg Zellen im Blut positiv mit reduzierter allergischer und Autoimmun-Erkrankung korreliert, so ist doch wenig über das Auftreten und die Rolle von Treg Zellen im Gewebe bekannt. Wir untersuchten betroffene Hautbiopsien von Patienten, die an atopischer Dermatitis oder anderen Erkrankungen litten, und fanden, dass obwohl zahlreiche IL-10 und TGF-β sekretierende Tr1 Zellen in der Dermis und Epidermis auftraten, keine $CD4^+CD25^+FoxP3^+$ vorhanden waren. Anschliessend führten wir Co-Kultur-Experimente mit beiden Treg Zelltypen in Kombination mit Keratinozyten und aktivierten Th1 Zellen durch. Ziel war es herauszufinden welche dieser Zellen, die normalerweise in der Lage sind die T Zellenaktivierung zu unterdrücken auch die Gewebsverletzungen in der atopischen Dermatitis unterdrücken können, die durch die Th1 Zellen–induzierte Keratinozyten-Apoptose hervorgerufen werden. Experimente mit Keratinozyten kombiniert mit T Zellen oder nur Zytokinen

zeigten, dass beide Typen regulatorischer T Zellen nicht in der Lage sind Effektorfunktionen von zuvor aktivierten T Zellen zu unterdrücken.

Zusammenfassend haben wir demonstriert, dass regulatorische T Zellen eine bedeutende Rolle bei der Erhaltung der Unempfänglichkeit von T Zellen für Allergene spielen. Die Induktion von Allergen-spezifischen Treg durch SIT könnte zu einem immunologischen Status führen bei dem das Zusammentreffen mit einem Allergen nicht mehr zu einer allergischen Reaktion führt. Wenn aber zuvor aktivierte T Zellen unter dem Einfluss eines Chemokin-Netzwerkes aktiv in das Gewebe einwandern dann können regulatorische T Zellen die zerstörerischen Effekte der Apoptose-induzierenden Effektor T Zellen nicht mehr unterdrücken.

5. Introduction

5.1 Allergy

Allergy is often seen as an illness that results from our changing lifestyle and environment. Although there is undoubtedly an increase in prevalence during the last five decades, allergic disease has probably existed as long as humanity. Possibly the earliest documented case of allergic disease in history dates back from some time between 3640 and 3300 BC, when the Egyptian pharaoh Menses died after being stung by a wasp. Another historic case is that of Britannicus (AD 41-55), son of the Roman emperor Claudius, who was allergic to horses and developed a rash and eye swelling to the extent that he could not see where he was going during official parades, reason enough to have to leave the throne to his stepbrother Nero. Both cases demonstrate how allergic disease can have a dramatic effect on people's lives.

In 1906, the Viennese paediatrician Clemens von Pirquet first used the term allergy to describe "an altered capacity of the body to react to a foreign substance". This broad term was later redefined as "disease following an immune response to an otherwise innocuous non-self antigen". Although often associated, the term atopy should not be confused with allergy. Atopy describes the increased susceptibility of an individual to developing an IgE-mediated immune response to antigens, which may lead to a type I immediate hypersensitivity response (table 1). However, not all allergic reactions are type I hypersensitivity responses. Both asthma and atopic dermatitis have non-atopic components and are a combination of immediate (type 1) and delayed (type IV) hypersensitivity. Allergic contact dermatitis is not IgE-mediated and is characterised by a type IV response, while allergic alveolitis and allergic vasculitis are both type III reactions [1].

Table 1. Hypersensitivity responses

	Type I	Type II	Type III	Type IV		
Immune reactant	IgE	IgG	IgG	Th1 cells	Th2 cells	CTL
Antigen	Soluble antigen	Cell- or matrix-associated antigen	Soluble antigen	Soluble antigen	Soluble antigen	Cell-associated antigen
Effector mechanism	Mast cell and basophil degranulation	FcR^+ cells (phagocytes, NK cells)	FcR^+ cells compliment	Macrophage activation	Eosinophil activation	cytotoxicity
Examples of hypersensitivity reaction	Allergic rhinitis, asthma, systemic anaphylaxis	Some drug allergies (e.g. penicillin)	Serum sickness, Arthus reaction	Contact dermatitis, tuberculin reaction	Chronic asthma, chronic allergic rhinitis	Contact dermatitis

Adapted from Immunobiology: the immune system in health and disease 5th edition, eds. CA Janeway Jr, P Travers, M Walport, MJ Slomchik. Garland Publishing, New York, NY, USA.

5.2 Cells and cytokines in allergic inflammation

In the 1980s, it was first discovered that separate populations of helper T cells could be distinguished on the basis of their cytokine secretion pattern. These subsets are derived from the same precursor, naïve $CD4^+$ T cells, and differentiate upon the first encounter of their specific antigen. The cytokines in the microenvironment, produced by antigen-presenting cells (APC) and resident tissue cells, determine the phenotype of the activated $CD4^+$ T cell. Traditionally, a distinction was made between only two types of $CD4^+$ T cells, but more recently a third population, the T regulatory (Treg) cells, has been described and defined. The generation of the right subset of T cells upon antigen encounter is crucial for an appropriate immune response. Domination of the wrong subset can lead to inadequate clearance of a pathogen, autoimmune disease or hypersensitivity.

Although T cells certainly make up the largest number of cells involved in the response against allergens, more cell types play an essential role in the correct outcome of an immune reaction. Both B cells and dendritic cells are increasingly seen as important determinants in the establishment of allergic inflammation or a healthy immune response. The role of these cells and their

mediators in the immune response against allergens will now be briefly discussed.

5.2.1 T helper 2 cells

T helper 2 (Th2) cells are a subset of T cells that secrete a particular set of cytokines, including IL-4, IL-5 and IL-13. Their development depends on the cytokine IL-4, which activates the transcription factors GATA-3 [2] and STAT6 [3]. The source that provides the initial IL-4 is still under debate, since Th2 cells themselves are the major producers of this cytokine. The principle function of Th2 is to induce IgE production by B cells/ plasma cells upon recognition of their specific antigen. The ligation of IgE to the high-affinity IgE receptor (FcεRI) on the surface of basophils and mast cells induces the release of inflammatory mediators, such as cytokines, leukotrienes and histamine. IgE and Th2 cytokines are thought to aid in the eosinophil-mediated immune response against helminth infections, but IgE also triggers the immediate hypersensitivity response to allergens.

5.2.1.1. Interleukin-4

Interleukin-4 (IL-4) is an 18 kD cytokine produced mainly by Th2 cells and also by activated mast cells and basophils. IL-4 induces Th2 differentiation from naive $CD4^+$ precursors, stimulates IgE production by B cells and mast cell/eosinophil mediated reactions and suppresses IFN-γ-dependent macrophage functions. In addition, IL-4 induces expression of the low-affinity IgE receptor (CD23) on B cells and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells. A reduced IgE synthesis has been shown in IL-4-deficient mice. IL-4 in combination with GM-CSF can be used to establish DC lines from adherent mononuclear cells, which express high levels of MHC molecules, CD1a, CD40, B7 and ICAM-1 [4]. The IL-4 receptor, CD124, shares a common β, and occasionally, γ chain with several other cytokine receptors.

5.2.1.2. Interleukin-5

Interleukin-5 (IL-5) is a 45-50 kD cytokine produced by Th2 cells and activated mast cells. IL-5 stimulates the growth and differentiation of eosinophils and activates mature eosinophils. IL-5 works in concert with IL-4; IL-4 increases IgE production, IgE opsonises helminths and binds eosinophils and IL-5 activates eosinophils to kill the parasites. IL-5-deficient mice have defective eosinophil responses and are therefore susceptible to helminth infections. IL-5 also stimulates B cell proliferation and IgA production. Anti-IL-5 monoclonal antibodies have been found to lower blood and sputum eosinophil numbers in humans and may therefore have a therapeutic potential for asthma and allergy [5].

5.2.1.3. Interleukin-13

Interleukin-13 (IL-13) is a 15 kD cytokine closely related to IL-4 and produced by Th2 cells, as well as mast cells and natural killer (NK) cells. IL-13 induces isotype switching on B cells to IgE. The main actions of IL-13 are to inhibit macrophage activation and to antagonise IFN-γ. IL-13 further stimulates mucus production by lung epithelial cells and may therefore also play a role in asthma. IL-13-deficient mice show a decreased IgE production and lower susceptibility to allergic reactions and asthma [6].

5.2.2 T helper 1 cells

The T helper 1 (Th1) subset of T cells is characterised by the secretion of IFN-γ. They are mostly generated upon infection with intracellular bacteria, mycobacteria and some parasites. These microbes are recognised by Toll-like receptors (TLRs) on the surface of macrophages and dendritic cells, which secrete IL-12 in response. Ligation of the IL-12 receptor on $CD4^+$ T cells activates the transcription factors T-bet [7] and STAT4 [8], and these promote the differentiation into Th1 cells.

The major Th1 cytokine, IFN-γ, stimulates macrophages to digest engulfed microbes, but also promotes the formation of non-inflammatory IgG4 class antibody against antigens. A Th1-dominated response against allergens is therefore sometimes seen as protective for allergic disease. Th1 cells however play an important role in delayed type hypersensitivity (type IV) reactions. For example, the IFN-γ they produce, in combination with the expression of FasL, is responsible for the major tissue injury event in AD, the apoptosis of keratinocytes and consequent spongiosis formation [9].

5.2.2.1. Interferon-γ

Interferon-γ (IFN-γ) is a classic Th1 cytokine and the principal activator of macrophages and has critical functions in innate and adaptive cell-mediated immunity. IFN-γ is also a crucial mediator of NK cell responses to intracellular pathogens. IFN-γ gene expression in T cells can be induced by IL-1, IL-2, IL-12, IL-18, TNF-α, antigens and mitogens, while IL-4, IL-10, corticosteroids, cyclosporine A and vitamin D inhibit IFN-γ expression. IFN-γ has an anti-viral activity, which is mediated by the induction of oxygen metabolites and nitric oxide in macrophages and neutrophils. Macrophages are activated by IFN-γ to kill tumour cells and intracellular parasites. IFN-γ influences T cell proliferation, differentiation and effector functions, by inhibiting Th2 cytokine expression and IgE synthesis. The receptor for IFN-γ is expressed on most cell types, thus providing a broad spectrum of effects.

5.2.3 T regulatory cells

T cells that are able to suppress the immune response were first described in the early 1970s [10]. This area of research was abandoned in the 1980s, when the mechanisms underlying immune suppression failed to be clarified, because of the insufficient analysis techniques available to researchers [11]. Exploration into the concept of T cell-mediated immune suppression strengthened once more in

the 1990s after new technological developments. Although the idea of Treg cells regulating immune responses via cell-cell interactions and/or the production of cytokines is currently well established, many aspects behind these mechanisms remain to be revealed. Various subpopulations of Treg cells, with different modes of operation, have been described (table 2). The three most common subtypes are discussed below.

5.2.3.1. Type1 regulatory cells (Tr1)

Tr1 cells, also known as adaptive or inducible Tregs, are defined by their ability to produce high levels of IL-10 with or without the production of TGF-β [12, 13] and are therefore also known as IL-10-secreting cells. They develop in the periphery under the influence of presumably immature dendritic cells and/or the presence of IL-10 and TGF-β [14]. A Tr1 cell subset can be generated *in vitro* by stimulating naive $CD4^+$ T cells in the presence of IL-10, IFN-α or a combination of IL-4 and IL-10 [12, 13]. A combination of dexamethasone and vitamin D3 can induce human and mouse naive $CD4^+$ T cells to differentiate *in vitro* into Tr1 cells [15], which, in contrast to previously described *in vitro* derived $CD4^+$ T cells, only produced IL-10, but no IL-5 or IFN-γ. Human IL-10-producing Tr1 cells are generated *in vivo* during the early course of allergen-SIT, implying that high and increasing doses of allergens induce Tr1 cells [16-18]. The near future holds the use of Tr1 cells as a novel target for developing new therapeutic agents, and as a cellular therapy for the induction of peripheral tolerance in allergy and autoimmunity [19-21].

5.2.3.2. $CD4^+CD25^+$ Treg cells

This subset of Treg cells, also known as the natural Tregs, accounts for 5–15% of peripheral blood $CD4^+$ T cells. They were the first type of Treg cell to be shown to inhibit the activation and proliferation of effector T cells [22-25]. The phenotype of these cells is not only characterised by the expression of the surface proteins CD4 and CD25 (IL-2Rα-chain), but also by the expression of the transcription factor FoxP3 [26]. They further express the surface markers cytotoxic T lymphocyte antigen-4 (CTLA-4) [27] and glucocorticoid-induced tumour

necrosis factor receptor (GITR) [28], which play a role in their suppressor activity. $CD4^+CD25^+$ Treg cells mostly develop in the thymus and in mouse models, depletion of these cells leads to hyperproliferation of normal lymphocytes and destruction of various tissues [29]. In humans, a mutation in FoxP3 leads to the rare immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome, which is associated with the development of atopic dermatitis and hyper IgE responses, as well as type 1 diabetes and other autoimmune diseases [30]. Although $CD4^+CD25^+$ Treg cells have been shown to inhibit the activation and proliferation of effector T cells *in vitro*, they may partially rely on the induction of Tr1 cells for their suppressive effect *in vivo* [31].

5.2.3.3. Th3 cells

These Treg cells are characterised by the production of high levels of TGF-β upon activation with antigen or anti-CD3 antibody [32]. Th3 cells were found to suppress encephalitis induction with myelin basic protein (MBP). TGF-β and IL-10 seem to play a crucial role, as neutralising antibodies against these cytokines abrogate the protective effects of these cells. *In vitro*, Th3 cells have been shown to exert bystander immune suppression.

Table 2. Types of regulatory T cells

Treg cell type	Suppressor mechanism
Tr1	IL-10, TGF-β
$CD4^+CD25^+$	IL-10, TGF-β, CTLA-4, PD-1, GITR
Th3	TGF-β
$CD8^+CD25^+CD28^-$	Same as $CD4^+CD25^+$
Qa-1-dependent $CD8^+$	Qa-1-specific TCR
$CD4^-CD8^-$	Induction of apoptosis
TCR γδ	IL-10, TGF-β

Adapted from Taylor A *et al*, Microbes Infect. 2005; 7:1049-55

5.2.4 B cells

As the only antibody-producing cell type, B cells play an important role in allergic inflammation. In type I hypersensitivity responses, they produce the IgE responsible for mast cell release of histamine and other inflammatory mediators, under the influence of Th2 cell cytokines. Apart from this pro-inflammatory role, a regulatory role for IL-10-secreting B cells has been recently proposed [33]. These B cells were shown to prevent the development of arthritis. Their suppressive effect is highly dependent on IL-10, since B cells isolated from IL-10-deficient mice fail to protect from arthritis. Helminth infection of mice has been shown to lead to an increased number of IL-10-producing B cells [34]. Upon adoptive transfer, these B cells protected mice from developing allergic hypersensitivity and anaphylaxis. In another mouse model, a deficiency in regulatory B cells was shown to correlate with an increased susceptibility to allergic airway inflammation [35].

5.2.5 Dendritic cells

Dendritic cells (DC) are the sentinels of the immune system, usually located at the interfaces of the body such as the skin and mucosa, and play an important role in the initiation and regulation of the immune response. They are considered the connecting link between allergen exposure and disease in the allergic form of atopic dermatitis (AD, see also section 5.3.1) [36]. Patients with allergic AD show a much higher expression of the high-affinity IgE receptor FcεRI on the two subtypes of DC in the skin, Langerhans' cells (LC) and epidermal dendritic cells (EDC), than healthy individuals or non-allergic AD patients [37]. Skin DC actively contribute in the shift from a Th2 to Th0/Th1 response, typical for AD, by the secretion of IL-12 and IL-18 upon ligation of FcεRI [38]. Similar to AD, dendritic cells have also been shown to play a critical role in the initiation of an allergic or a healthy response against aeroallergens in the lung [39].

There are some indications that DC can induce peripheral T cell tolerance and that a regulatory DC subset may exist. Pulmonary dendritic cells from mice

exposed to respiratory antigen transiently produce IL-10 [40]. These phenotypically mature pulmonary $B7^{hi}$ DC stimulate the development of $CD4^+$ Tr1-like cells that also produce high amounts of IL-10. Adoptive transfer of pulmonary DC from IL-$10^{+/+}$, but not IL-$10^{-/-}$, mice exposed to respiratory antigen induced antigen-specific unresponsiveness in recipient mice. Accordingly, IL-10 inhibits the development of fully mature DC, which induces a state of alloantigen-specific anergy in $CD4^+$ T cells [41]. These studies show that IL-10 production by DC is critical for the induction of tolerance, and that phenotypically mature DCreg may exist under certain circumstances.

5.3 Allergic disease

The exact reason why some individuals develop allergic immune responses to common environmental antigens, while others tolerate these, is not known. However, several options have been suggested in the recent past. Studies have uncovered various susceptibility genes that increase the risk of developing asthma or atopic disease within certain families. For example, single nucleotide polymorphisms in the *IL18* gene have been suggested to relate to the development of atopic eczema, in particular to the intrinsic subtype [42]. The *gly16* allele of the β2-adrenergic receptor gene predisposes to nocturnal asthma, but not to bronchial hyperresponsiveness or mild asthma [43]. Furthermore, variations in the gene for T cell immunoglobulin mucin (TIM)-1, but not TIM-3, were found to contribute to asthma susceptibility in an African-American population [44]. Variations in chemokine genes have also been associated with allergen susceptibility, as in the case of the haplotype block encoding several CXC chemokines [45]. Based on these findings, it seems that genetic predisposition can influence an individual's atopic status. It should however be kept in mind that this higher susceptibility is often of limited magnitude and that each variant is only found in minor fractions of patients. Furthermore, it does not explain the increasing prevalence of allergic disease.

Apart from genetic factors, a major factor that has been found responsible for the drastic increase in the prevalence of allergy in western society is our changing environment. The rapid industrialisation in the western world during the second half of the last century has brought along polluting substances, such as diesel exhaust particles, nitrogen oxide, ozone and tobacco smoke, which might be associated with the exacerbation and severity of asthma [46]. However, their role in the induction of allergy remains controversial.

In contrast to the outside milieu, our homes and living areas have become cleaner and more devoid of microbes during the last few decades. The original hygiene hypothesis suggested that a decreased microbial exposure early in life leads to the failure of the immune response to shift from a T helper (Th)2 cell-dominated to a Th1 cell response. A widely used example of the effects of the living environment during childhood on the development of allergies is that of farmers' children. Multiple studies, including a very elaborate one among over 700 children in Finland [47], have shown a significantly lower incidence of allergy in children growing up on a farm, compared to those growing up in an urban environment. This remarkable difference is often attributed to the higher microbial load encountered on the farm. Other observations related to microbial infection early in life include the increased risk of childhood atopy after the use of antibiotics and the negative correlation with fever episodes during childhood [48, 49]. These recent findings on the influences of our changing life style, although in some cases not more than suggestive, support the hygiene hypothesis and would indeed help explain the increase of Th2-related diseases, such as allergy, during the past decades. However, the simultaneous rise of Th1-related autoimmune diseases such as Crohn's disease [50] and type 1 diabetes [51] during this same period remains to be explained. Therefore, one might suggest that reduced microbial exposure does not result in a dysbalance between Th1 and Th2 cells, but rather results in the aberrant expression of both subsets of T cells.

5.3.1 Atopic dermatitis

Atopic dermatitis (AD) is one of the most frequent inflammatory skin diseases in the western world. AD is generally characterised by severe pruritus (itchiness), eczematous lesions, elevated total serum IgE, and specific IgE to aeroallergens, food allergens, auto-antigens and bacterial superantigens. In 1980, Hanifin and Rajka established diagnostic criteria, which are still used in clinical practice today [52]. A task force of the European Academy of Allergology and Clinical Immunology (EAACI) proposed a new nomenclature in 2003 [53], but the new term atopic eczema (AE) has not been universally adopted.

Although termed atopic, AD is not always associated with elevated serum IgE or specific IgE. Depending on populations and criteria, different studies have reported this to be the case in 10% to 45% of all AD patients [54]. Therefore, AD is often divided into two different categories:

1) The extrinsic or allergic form (EAD) is characterised by the elevation of total serum IgE levels. EAD might involve only the skin, but is often accompanied by comparable inflammation of the lung and nasal mucosa [55]. About half of all individuals diagnosed with EAD also develop asthma and two-thirds develop allergic rhinitis. The cutaneous manifestations of EAD often begin in early childhood and are followed by IgE-mediated sensitisation to food allergens and the subsequent development of hypersensitivity to inhaled allergens. This development of IgE-mediated diseases throughout life is also known as the atopic march.

2) The intrinsic or non-allergic form (IAD) shows normal total IgE serum levels and has negative serum levels of allergen-specific IgE [54]. This subtype of AD is further characterised by a similar clinical phenotype to EAD and negative type 1 skin hypersensitivity to food allergens and aeroallergens.

Despite these differences, the tissue injury in both forms seems to result from a common immunological mechanism (Figure 1). In both EAD and IAD, T cells activate B cells upon allergen recognition. In EAD this leads to IgE production, while in IAD IgG is produced [56]. Activated Th2 cells upregulate the

expression of cutaneous lymphocyte-associated antigen (CLA), the homing receptor involved in selective migration of T cells to the skin. In the dermal layer of the skin, these T cells subsequently upregulate the secretion of interferon-γ (IFN-γ) as well as the expression of the death receptor Fas and its ligand. This is induced through the local production of IL-12 and IL-18 by resident Langerhans' cells, epidermal dendritic cells (EDC) and keratinocytes and gives the T cells a Th0/Th1 phenotype [57-59]. Accordingly, T cells from draining lymphatic vessels of the skin, which represent de-homing effector T cells, express CLA and elevated levels of IFN-γ [60]. The T cells themselves are protected from apoptosis by cytokines and contact with the extracellular matrix (ECM) [61], although they express both Fas and FasL. Contact with the ECM has been shown to be essential for the survival of anchorage-dependent cells, but also appears to rescue T cells (which are not anchorage-dependent) from apoptosis. Furthermore, the common γ chain shared by several cytokine receptors is an essential signalling component for T cell survival [62]. While T cells are protected from apoptosis, IFN-γ and FasL cooperate to induce the major tissue injury event in AD, the apoptosis of keratinocytes. In asthma, a similar event leads to shedding of the epithelium, but in AD, keratinocyte apoptosis leads to the formation of spongiosis in the epidermal layer. It seems likely that keratinocyte stem cells located at the basal membrane are protected from T-cell-induced apoptosis due to strong anti-apoptotic signals from dermal fibrocytes and ECM proteins [63].

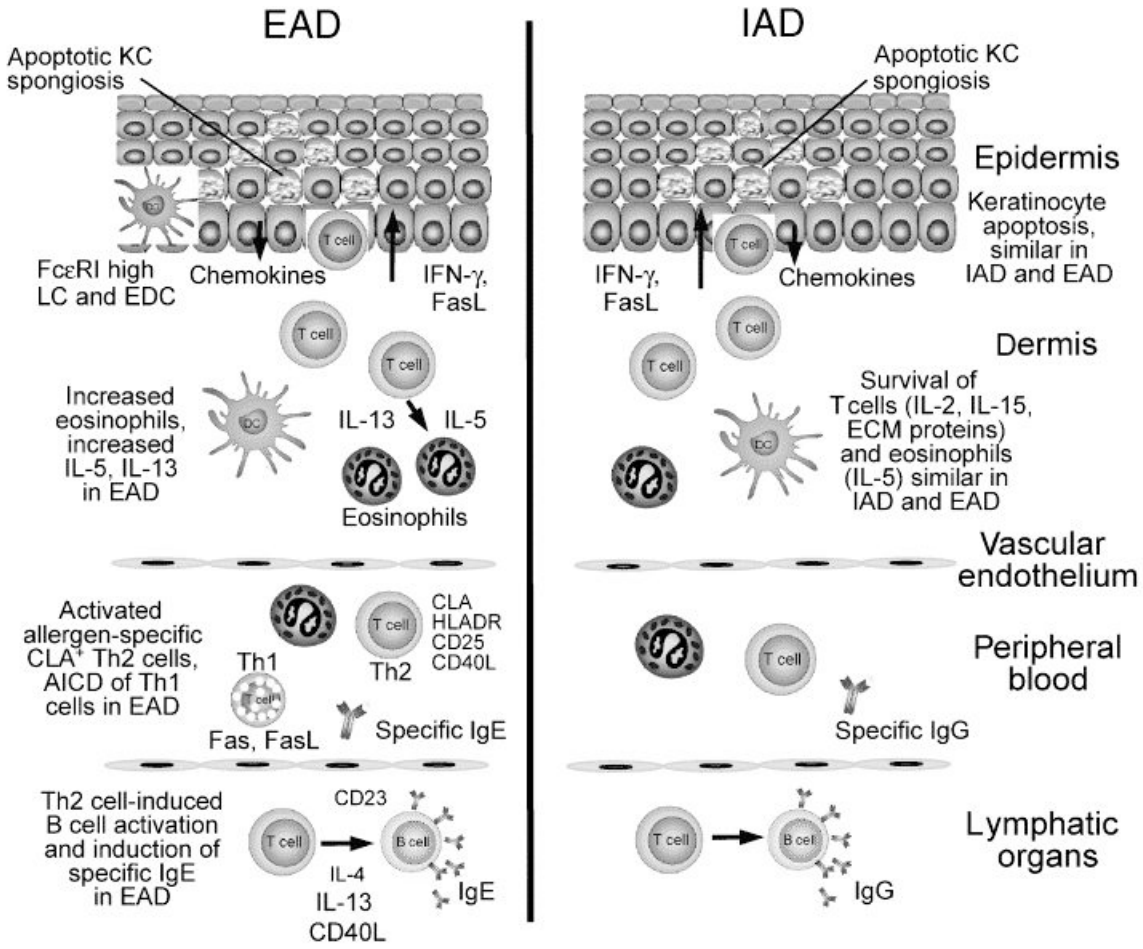

Figure 1. Immune effector mechanisms in extrinsic (EAD) and intrinsic (IAD) atopic dermatitis. In the peripheral blood of EAD patients $CLA^{+}CD45RO^{+}$ T cells are activated and the Th1 cells in particular undergo Fas-mediated activation-induced cell death (AICD). In contrast, T cells infiltrating the skin do not go into apoptosis and are saved by cytokines and by contact with the ECM. These T cells show elevated levels of IFN-γ, which induces apoptosis of keratinocytes. In EAD, T cells in the skin secrete IL-5 and IL-13, which prolongs eosinophil survival. In IAD, no IgE, but IgG against allergens is found in the circulation. Langerhans' cells (LC) and epidermal dendritic cells (EDC) express high levels of FcεRI in EAD. From Akdis CA and Akdis M, Clin. Exp. Allergy. 2003; 33:1618-21

5.4 T cell regulation in health and disease

5.4.1 T regulatory cells and the control of disease

Numerous animal and human studies have shown that Treg cells can actively suppress the development of autoimmune disease and immunity against alloantigens. Sakaguchi *et al.* showed for first time that depletion of regulatory T cells from mice led to the development of autoimmune disease [64]. Co-transfer of $CD4^+CD25^+$ Treg cells together with effector, disease-causing, T cells prevented mice from developing multiple experimentally induced autoimmune diseases such as colitis, gastritis, type 1 diabetes and thyroiditis [29, 65-67]. In human studies, the role of regulatory T cells is mostly demonstrated by a lower frequency or activity of Treg cells compared to unaffected individuals. In multiple sclerosis, for example, $CD4^+CD25^+$ Treg cells from patients have a markedly reduced suppressive capacity compared to healthy individuals [68]. Patients with chronic graft-versus-host disease (GVHD) show reduced numbers of $CD4^+CD25^+$ Treg cells compared to transplantation patients without GVHD [69]. Equally, adoptive transfer of donor $CD4^+CD25^+$ Treg cells after haematopoietic cell transplantation promoted engraftment and tolerance in a mouse model of GVHD [70]. $CD4^+CD25^+$ Treg cells activated by allogeneic APCs have been found to induce long term tolerance to bone marrow grafts, but simultaneous rejection of third-party bone marrow still occurred, indicating that *in vivo* $CD4^+CD25^+$ Treg cells work antigen-specifically [71]. Somerset *et al.* showed an increase in circulating maternal $CD4^+CD25^+$ Treg cells during early pregnancy [72]. Polanczyk *et al.* reported that oestrogen augments FoxP3 expression by $CD4^+$ T cells, suggesting that oestrogen aids in the formation of $CD4^+CD25^+$ Treg cells, which can suppress allogenic immune responses against the growing foetus [73]. In concordance with these observations, cases of miscarriage and spontaneous abortion show a low number of circulating $CD4^+CD25^+$ Treg cells, suggesting a role for these in successful pregnancy [74].

Although Treg cells obviously have a benign regulatory role in many conditions, immune suppression can also lead to serious illness. Already twenty-five years ago, it was first recognised that the adoptive transfer of tumour-reactive T cells was only beneficial in T cell-deficient mice, and not in immunocompetent animals, suggesting a presence of regulatory cells in tumour-bearing individuals [75]. Several studies have since demonstrated an increased immune response against tumours after depletion of $CD4^+CD25^+$ Treg cells [76, 77]. An upregulation in the number of circulating tumour antigen-specific $CD4^+CD25^+$ Treg cells has been shown in human studies of various forms of cancer [78].

The long-term persistence of pathogens in a host is equally associated with the presence of antigen-specific $CD4^+CD25^+$ Treg cells. In the case of the parasite *Leishmania major*, for example, $CD4^+CD25^+$ Treg cells accumulate in the dermis of the skin and suppress anti-parasite immune responses by effector T cells [79]. Also in tuberculosis, increased levels of $CD4^+CD25^+$ Treg cells have been found at the site of infection and in the blood of patients, compared to healthy individuals [80].

Even though Treg cells may play a destructive role in cancer and chronic infectious diseases[81-86], clinical studies are required to demonstrate whether *in vivo* generation or adoptive transfer of Treg cells and/or their related suppressive cytokines can be used to alter the progression of allergy and asthma. The generation of Treg cells and the enhancement of their suppressive properties provide important targets not only for use in allergy and asthma, but also in transplantation- and autoimmunity.

5.4.2 Allergen-specific immunotherapy

Allergic disease generally persists lifelong, although examples of spontaneous remission of symptoms do exist. The most well known example is probably the “outgrowing” of childhood asthma. More than 20% of asthmatic children were found to be in complete remission by the age of 32-42, without ever undergoing

immunotherapy [87]. Cow's milk allergy is often outgrown rapidly and a short milk-free period in children's diet often suffices to change the immune response to milk antigens [88]. Despite these remarkable observations, there is still great need for curative treatment of allergic disease. The only currently available therapy with long-lasting effect is allergen-specific immunotherapy (SIT). Allergen-SIT has been commonly used as a treatment for allergic diseases for almost a century. Although results tend to vary, many examples of successful allergen-SIT exist. The success of the therapy depends on patient selection, the time of onset, accompanying diseases, the severity of atopic status, the way of administration, and the type of vaccine used.

5.4.2.1. Conventional Immunotherapy

Allergen-SIT commonly involves the intradermal injection of allergen extracts. Immunotherapy against insect venom allergies has become very common and many studies on successful allergen-SIT have been reported within this field. The adverse effects of the most common way of venom-SIT, with aqueous venom solutions, are however often a reason for patients to discontinue treatment. Conventional allergen-SIT has been performed for almost a century using various types of allergen extracts. Despite the long-lasting success of conventional immunotherapy, there is great need for vaccines with higher efficacy and safety. The allergen mixtures in use often contain toxic or non-allergenic protein and can therefore cause adverse effects. Furthermore, allergen-SIT can induce new IgE reactions or in rare cases even anaphylaxis in response to allergens contained in the extract. A recent survey in North America investigated the number of fatalities as a result of allergen-specific immunotherapy between 1990 and 2001 [89]. Their approximation of 3.4 deaths per year in North America alone clearly demonstrates the need for improved allergen-SIT vaccination strategies.

5.4.2.2. Novel Immunotherapeutic Strategies

Several possibilities have been explored to comply with the need for novel, safer and more efficient, vaccination strategies. One approach is to use non-injection routes of administering allergen, such as oral immunotherapy, local bronchial immunotherapy, local nasal immunotherapy or sublingual immunotherapy. Oral administration (swallowing) of allergens has been used as a therapy since 1927. However, most non-injection routes of administration have not led to an adequate alteration of the immune response. Only sublingual therapy is still in clinical use today, with successful treatment reported in cases of allergic rhinitis and asthma [90].

Another strategy to improve allergen-SIT involves the vaccination with allergen-encoding DNA. Occasionally, experimental therapies with intradermal or intramuscular injection of allergen-encoding DNA have shown reduced levels of IgE, accompanied by a shift in allergen-specific T cell balance from Th2 to Th1 [91]. A lack of potency of DNA vaccines in humans has been reported not only in allergen-specific DNA vaccines, but also in the fields of infectious disease and cancer [92].

More studies have focused on the use of well-defined hypoallergenic peptides instead of the full natural allergen protein. The benefit of peptide immunotherapy (PIT) is based on the idea that short allergen peptides, either native sequences or altered peptide ligands with amino acid substitutions, still contain the epitopes for recognition by T cells, but without the possibility of IgE cross-linking which induces anaphylaxis [93]. Successful PIT using exact T cell epitope peptides from the major allergen phospholipase A2 has been reported several years ago [94]. Peptides that can be used for PIT have now been defined for various allergens, including the latex allergen Hev b 6.01, the cat allergen Fel d 1, the house dust mite allergen Der p 1 and birch pollen allergen Bet v 1 [95-98]. Vaccination with these peptides generally showed a reduced Th2 response and often an increase in the secretion of the immunosuppressive cytokine IL-10, suggesting a shift towards Tr1 cells. Since peptide vaccination does not induce an IgE response, the therapy is well tolerated by patients. These results suggest

that PIT might be a suitable way of allergen-SIT. One down point of PIT, however, might be a possible limited applicability, since not all patients will respond to the same peptides, due to the diversity of MHC class II-mediated antigen presentation.

Another very recent development in the immunisation with allergens without inducing IgE responses is gaining attention. It uses the previous observation that IgE recognises the three-dimensional structure of allergens, while T cells recognise linear sequences [99]. Kussebi *et al.* genetically engineered a fusion protein that contains T cell epitopes, but not B cell epitopes, of two major bee venom allergens, phospholipase A_2 (PLA) and hyaluronidase [100]. Karamloo *et al.*, with a similar approach, combined three major bee venom allergens, PLA, hyaluronidase and melittin [101]. Pre-treatment with either of the fusion proteins protected mice from IgE, IgG2a and IgG1 antibody responses to later immunisation, therefore suggesting the induction of allergen-specific tolerance. Another group has recently shown that a polypeptide of three recombinant grass pollen allergens, Bet v 1, Phl p 1 and Phl p5, had comparable protective effects [102]. Human immunotherapy with a combination of five recombinant grass pollen allergens successfully led to the amelioration of clinical symptoms of allergic rhinitis, with a shift in the immune response from IgE to IgG4 and the induction of IL-10-secreting Treg cells [103]. The wide applicability of fusions, hybrids and chimeras of allergens, which preserve T cell tolerance-inducing capacity without IgE binding, make these a very promising tool for allergen-SIT as well as preventive allergy vaccination.

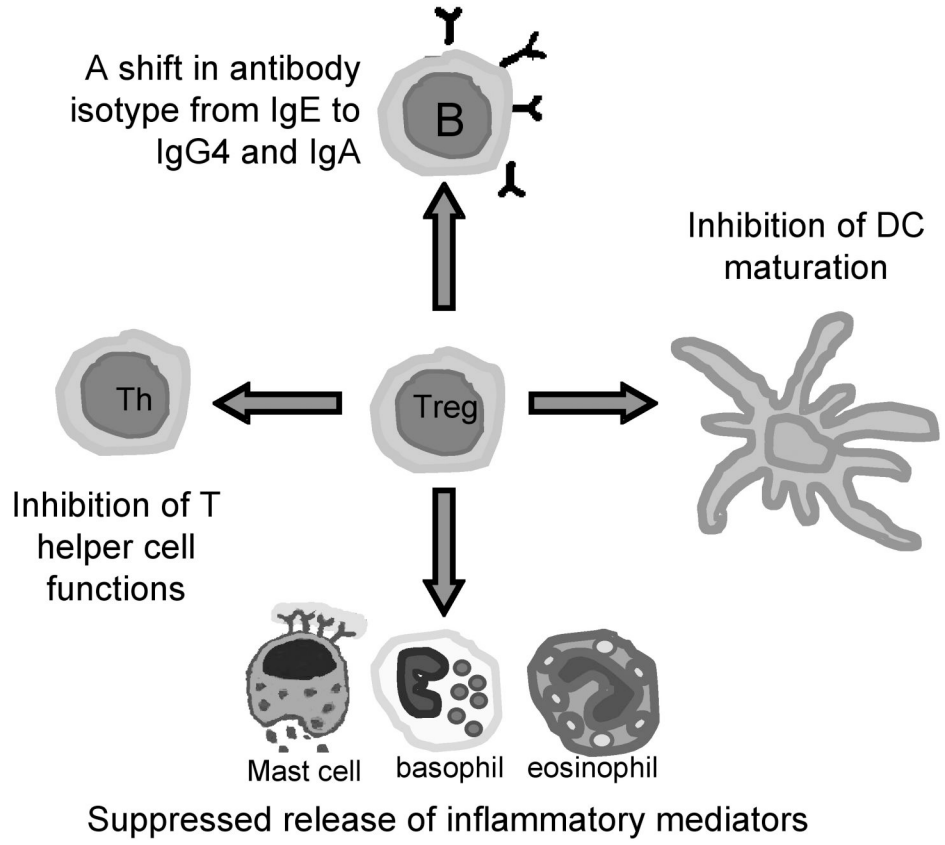

Figure 2. The role of Treg cells in immunotherapy. Treg cells control the activation of both Th1 and Th2 cells. Furthermore, their cytokines IL-10 and TGF-β induce a shift from allergen-specific IgE towards IgG4 and IgA, respectively, and inhibit the maturation of dendritic cells. Also, their activation leads to a reduction in the cytokines required for the release of inflammatory mediators by various effector cells. Adapted from Verhagen J *et al.*, Int. Rev. Immunol. 2005; 24:533-48

5.4.2.3. Treg cells in allergen-specific immunotherapy

The induction of peripheral T cell tolerance plays a crucial role in allergen- SIT [16, 17, 20, 104]. Mouse studies have shown protection from the development of allergen-induced Th2 responses after the application of *in vitro* engineered allergen-specific Treg cells [105]. Similarly, the proliferative response to nickel of $CD4^+$ T cells from healthy, nonallergic individuals was strongly augmented when Treg cells were depleted [106]. Numerous studies in humans and mice have shown

an increase in regulatory T cells in peripheral blood during allergen-SIT. These do not only include increased numbers of $CD4^+CD25^+$ Treg cells, but also the levels of IL-10-secreting Tr1 cells have been shown to be upregulated during the course of therapy [84, 107]. The suppressive actions of these Tregs could be blocked by the use of neutralising mAbs against IL-10 and TGF-β [84]. The appearance of $CD4^+CD25^+$ Treg cells has been associated with the spontaneous remission of cow's milk allergy [88]. Furthermore, it has been shown that $CD4^+CD25^+$ Treg cells from atopic donors have a reduced capability to suppress the activation of $CD4^+CD25^-$ effector T cells [108].

A shift from Th2 to Th1 responses is sometimes reported in studies of allergen-SIT [90]. Although Th1 cells counteract the effects of allergy-inducing Th2 cells, a Th1 response against allergens is equally undesired. Furthermore, IFN-γ is not required for induction of aerosol-induced IgE unresponsiveness, as shown in IFN-γ knockout mice and wild-type mice treated with anti-IFN-γ antibody, showing that there is no requirement for Th1 cells in successful allergen-SIT. Moreover, neither $CD8^+$ T cells nor γδ TCR T cells are essential for a reduction in IgE production [109], therefore further suggesting a major role for Treg cells.

T cell tolerance is initiated by the autocrine action of IL-10 and TGF-β, which are produced in significant amounts by Treg cells [16, 17, 20]. A phenomenon crucial for successful allergen-SIT is the shift in balance between IgE and IgG4. Although peripheral tolerance has been demonstrated in specific T cells, the capacity of B cells to produce specific IgE and IgG4 antibodies is not eliminated during allergen-SIT [104]. In fact, during the early phase of treatment, specific serum levels of both isotypes increase. The increase of specific IgA and IgG4 in serum coincides with increased TGF-β and IL-10 respectively.

Although IgE antibody levels and IgE-mediated skin sensitivity normally require several years of SIT to show a reduction, most patients are already protected against allergens at an early stage of allergen-SIT. This is probably because suppressed B cells and Treg cells cannot sufficiently provide the cytokines that effector cells (mast cells, basophils and eosinophils) require for priming, survival and activity [110, 111]. Allergen-SIT efficiently modulates the mast

cell and basophil activation thresholds and decreases IgE-mediated histamine release [112, 113]

5.5 Treg cells: suppressor mechanisms in allergic inflammation.

5.5.1 Surface molecules: CTLA-4, PD-1 and GITR

$CD4^+CD25^+$ Treg cells appear to suppress effector T cells in a cell contact-dependent manner *in vitro* [114]. Three major surface molecules have been associated with the function and activity of $CD4^+CD25^+$ Treg cells. The first, CTLA-4, is a co-stimulatory receptor of the CD28 family and a negative regulator of T cell activation [115]. CTLA-4 is consistently highly expressed on human and murine $CD4^+CD25^+$ Treg cells [27]. Similarly, it has been reported that the suppressive capacity of $CD4^+CD25^+$ Treg cells strongly correlates with high levels of CTLA-4 expression, consistent with a role in the suppressive function of $CD4^+CD25^+$ Treg cells [116]. $CD4^+CD25^+$ Treg cells from CTLA-4-deficient mice show an impaired suppressive capacity [117] and these animals develop a fatal lymphoproliferative disease [118]. A convincing *in vitro* demonstration of a direct functional role of CTLA-4 in Treg-mediated suppression, however, remains. CTLA-4-mediated regulation of $CD4^+CD25^+$ Treg cells is most likely due to an effect on their suppressive function, be it either direct or via the production of a secondary inhibitor [27].

Programmed death-1 (PD-1) is another receptor that negatively regulates T cell activation. Like CTLA-4, it is a member of the CD28 family of co-stimulation receptors, but it has its own set of B7 family ligands, PD-L1 and PD-L2. An inhibitory role for PD-1 has been suggested based on the development of autoimmune disease in PD-1-deficient mice [119]. Treatment of mouse target T cells with anti-PD-L1 led to the requirement of higher ratios of $CD4^+CD25^+$ Treg cells to suppress T cell activation [120].

Alloantigen-specific $CD4^+CD25^+$ Treg cells abundantly express GITR [28]. This acts as a negative regulator of $CD4^+CD25^+$ Treg cell suppression, since binding of GITR by soluble GITR ligand leads to abrogation of the natural anergic state of the cell [121]. It may therefore be a target for immunotherapy in cases of excessive $CD4^+CD25^+$ Treg cell function, as in cancer or chronic infection.

5.5.2 Interleukin-10

Studies have shown lower levels of IL-10 in the bronchoalveolar-lavage fluid of asthmatic patients than in healthy controls, and a lower production of IL-10 mRNA in T cells of asthmatic children than of healthy children [122, 123]. These findings indicate that an increase in IL-10 production might lead to a decrease in allergic reactions. Reactivation of tolerised T cells can result in the distinct production of either Th1 or Th2 cytokine profiles depending on cytokines present in the tissue microenvironment [104]. Antigen presentation to anergic T cells in the presence of IL-2 or IL-15 fully restores their ability to secrete IFN-γ, but not IL-4. IL-15 is secreted by APC and many tissue cells and may therefore act to recover Th1 cells, but not Th2 cells, after allergen-SIT. Furthermore, IL-10 has been reported to block the B7/CD28 costimulatory pathway. As a result of this, IL-2 produced in the microenvironment of peripheral tissues can restore allergen-specific T cell activation, without the induction of IL-5 production. These findings suggest that IL-10, in combination with microenvironmental factors, can deviate the immune response against allergen from a Th2 to a Th1 biased mechanism.

In the early stages of allergen-SIT, serum IgE levels do not immediately show a marked decline, but the increase in antigen-specific IgG4 is more striking and the ratio of specific IgE to IgG4 decreases by 10- to 100-fold [17]. A similar change in specific isotype ratio was observed in SIT of various allergies. Meanwhile, IL-10 is induced and progressively secreted during SIT and appears to counter-regulate antigen-specific IgE and IgG4 antibody synthesis [16]. IL-10 has two major effects on B cells, which could result in the shift in balance as

observed. When added to PBMC during the first 3 days of in vitro culture, IL-10 decreases ε transcript expression, and therefore production of IgE. However, when added to already committed B cells, IL-10 induces further upregulation of IgE production. Furthermore, IL-10 enhances γ4 transcript expression and IgG4 production induced by IL-4 [124]. Although IL-10 is not a switch factor for IgG4, it may augment IgG4 production by favouring the differentiation/activation of IgG4-secreting cells or by enhancing the IL-4-induced class switching.

The molecular mechanisms of T cell suppression by IL-10 have been investigated intensively. IL-10 has been found to inhibit the proliferative T cell response in PBMC to various antigens, and the superantigen staphylococcal enterotoxin B [125]. IL-10 does not affect the proliferative responses of anti-CD3-stimulated T cells, but significantly inhibits the anti-CD28-induced proliferation. The investigation of triggered TCRs on T cells demonstrated the requirement for co-stimulation in T cell activation and its relation to the number of triggered TCRs [125]. T cells stimulated with variable concentrations of anti-CD3 mAb, and a constant amount of anti-CD28 showed that low numbers of triggered TCRs required CD28 co-stimulation and that only this co-stimulatory effect could be inhibited by IL-10. The stimulation of CD28 by specific monoclonal antibodies induces tyrosine phosphorylation of the cytoplasmic domain of the receptor. Ligation of IL-10 receptor (IL-10R) at the time of mAb stimulation inhibits the tyrosine phosphorylation of CD28 within minutes [125, 126]. The inhibitory effect of IL-10 appears to be specific for the co-stimulation pathways, as IL-10 does not affect ZAP-70 tyrosine phosphorylation stimulated by CD3 cross-linking [125]. As a consecutive event for signal transduction, phosphatidylinositol 3-kinase (PI3-K) binds to tyrosine phosphorylated co-stimulatory molecules by its p85 subunit. This association between co-stimulatory receptors and the PI3-K p85 subunit is inhibited by IL-10. IL-10 exerts its biological functions through the activation of Jak1 and Tyk2, the members of the receptor-associated Janus tyrosine kinases family and Stat1 and Stat3, and in certain cells Stat5 [127]. Previous studies demonstrated that IL-10 does not only inhibit T cells, it is also a potent inhibitor of activated monocytes and macrophages [128]. Since monocytes and macrophages

do not express CD28, the inhibitory impact of IL-10 is likely to occur through other mechanisms in non-T cells. In monocytes, IL-10 was shown to induce expression of the suppressor of the cytokine-signalling-3 (SOCS3) gene that may play a role in the inhibition of IFN-induced tyrosine phosphorylation of Stat1 [129].

Treg-derived IL-10 can have many other effects on the immune response, as well. The presence of IL-10 during maturation of DC can inhibit their full maturation, leading to decreased expression of MHC class II and several adhesion and co-stimulatory molecules. This results in the long lasting induction of tolerance in both Th1 and Th2 cells. In addition, IL-10 has been shown to reduce pro-inflammatory cytokine release from mast cells [130], down-regulate eosinophil function and activity, and suppress IL-5 production by resting human Th0 and Th2 cells [131].

5.5.3 Transforming growth factor-β (TGF-β)

The role of TGF-β in the control of atopic disease has long been unclear, but is now gradually being unveiled. TGF-β-deficient mice have been demonstrated to show a higher susceptibility to asthma-like lung inflammation than their healthy counterparts [132]. Furthermore, mice deficient in the transcription factor RUNX3 (a major mediator of TGF-β signalling) also show the spontaneous development of asthma-like symptoms [133]. A mouse model of food allergy showed a significant reduction of secreted IgA antibodies in the gut. The recovery of this was correlated to the local production of TGF-β [134].

Several reports have shown the importance of TGF-β in the induction and functioning of $CD4^+CD25^+$ Treg cells. $CD4^+CD25^+$ Treg cells not only express surface membrane-bound TGF-β in its latent form [135], but also express TGF-β in its active configuration on the cell surface [136]. One animal model showed that $CD4^+CD25^+$ Treg cells from wild-type mice, but not those from mice with impaired TGF-β signalling could suppress colitis in wild-type mice [137]. They further demonstrated that TGF-β promotes the expansion of $CD4^+CD25^+$ Treg

cells. Marie *et al.* [138] also showed that TGF-β maintains the FoxP3 expression of $CD4^+CD25^+$ Treg cells. In a model of autoimmune diabetes, it was shown that the onset of diabetes was slowed down by TGF-β-dependent $CD4^+CD25^+$ Treg cells, but also that a progressive resistance to TGF-β-mediated suppression played a role [139]. Furthermore, experimental tracheal eosinophilia was inhibited by the induction of TGF-β-secreting $CD4^+$ T cells [140].

Less is known about the molecular mechanisms behind TGF-β-mediated suppression in allergen-SIT. High levels of TGF-β have been demonstrated to downregulate the expression of FcεRI on Langerhans cells [141], leading to reduced IgE-mediated allergen presentation. Furthermore, it has been shown that the increased allergic inflammation seen after blocking CTLA-4 is clearly associated with decreased TGF-β levels within the bronchoalveolar-lavage fluid [142]. Finally, a link has been suggested between TGF-β and the induction of an IgA-dominated antibody response in mice [134].

6. Results

6.1 Absence of T regulatory cell expression and function in atopic dermatitis skin.

J allergy Clin Immunol., 2006. 117: 176-183

Absence of T-regulatory cell expression and function in atopic dermatitis skin

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Background: The role of regulatory T cells has been widely reported in the suppression of T-cell activation. A dysfunction in $CD4^+CD25^+$ T-regulatory cell–specific transcription factor FoxP3 leads to immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome, often associated with atopic dermatitis. Increasing the number and activity of regulatory T cells in affected organs has been suggested as a remedy in various inflammatory diseases, including allergy.
Objective: To determine the presence and function of regulatory T cells in atopic dermatitis.
Methods: Immunohistochemistry of lesional atopic dermatitis skin and control skin conditions was used to demonstrate regulatory cells and cytokines in situ. The role of effector and regulatory T cells as well as their specific cytokines in apoptosis in human keratinocyte cultures and artificial skin equivalents was investigated.
Results: Human T-regulatory type 1 cells, their suppressive cytokines, IL-10 and TGF-β, as well as receptors for these cytokines were significantly expressed, whereas $CD4^+CD25^+FoxP3^+$ T-regulatory cells were not found in lesional and atopy patch test atopic dermatitis or psoriasis skin. Both subsets of regulatory T cells suppress the allergen-specific activation of T_H1 and T_H2 cells. In coculture and artificial skin equivalent experiments, subsets of T-regulatory cells neither induced keratinocyte death nor suppressed apoptosis induced by skin T cells, T_H1 cells, IFN-γ, or TNF-α.
Conclusion: A dysregulation of disease-causing effector T cells is observed in atopic dermatitis lesions, in association with an impaired $CD4^+CD25^+FoxP3^+$ T-cell infiltration, despite the expression of type 1 regulatory cells in the dermis. (J Allergy Clin Immunol 2006;117:176-83.)

Key words: *Regulatory T cell, atopic dermatitis, apoptosis, suppression, regulation, skin, human, inflammation*

Abbreviations used
AD: Atopic dermatitis
APT: Atopy patch test
FasL: Fas ligand
HDM: House dust mite
NAD: Nonallergic type of dermatitis
Tr1: T-regulatory type 1
Treg: T-regulatory

Atopic dermatitis (AD) is a chronic relapsing skin disorder with an interplay of migrating lymphocytes and epidermal keratinocytes (KC).[1,2] Lesional AD skin is histologically characterized by dermal mononuclear infiltration and spongiosis in the epidermis. At the initial stages of inflammation, T_H2 cells migrate to the dermis, where they acquire a T_H0/T_H1 phenotype under the influence of IL-12, produced by antigen-presenting cells or activated keratinocytes.[3-5] These T_H0/T_H1 cells are characterized by the expression of Fas ligand (FasL) and secretion of significant amounts of the effector cytokines TNF-α and IFN-γ.[2,4,5] The secreted IFN-γ induces apoptosis of keratinocytes, leading eventually to the eczematous lesions characteristic of AD.[6,7] In response, keratinocytes upregulate the production of IFN-γ–inducible chemokines,[8] which in turn promotes the further infiltration of T cells into the epidermis, thereby augmenting the severity of inflammation and keratinocyte apoptosis.

After their initial discovery in the early 1970s, the concept of T-regulatory (Treg) cell–mediated immune suppression has been extensively explored. Two main groups of Treg cells have been defined. One comprises the natural Treg cells, which are characterized by their $CD4^+CD25^+$ phenotype. These cells have been suggested to develop under the control of the transcription factor FoxP3.[9] The other group of Treg cells, the adaptive Treg or T-regulatory type 1 (Tr1), are characterized by the secretion of high levels of IL-10 with or without TGF-β.[10-12] They develop in the periphery under the influence of presumably immature dendritic cells[13] and/or the presence of IL-10 and TGF-β, but also immunosuppressive drugs like glucocorticoids and vitamin D3,[14] and operate in a cytokine-mediated manner.

Most research on the inhibitory capacities of Treg cells has focused on their ability to suppress proliferation of effector T cells. It has been tempting to speculate that migration of increased numbers of Treg cells to the inflammation area, or the induction of their local proliferation, might be beneficial in the treatment of several

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Authors' laboratories supported by Swiss National Science Foundation grants No. 31-105865 and 32-100266 and the Global Allergy and Asthma European Network (GA2LEN).
Received for publication July 11, 2005; revised October 20, 2005; accepted for publication October 28, 2005.

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0091-6749/$32.00

doi:10.1016/j.jaci.2005.10.040

inflammatory diseases, including allergy, autoimmunity, and transplantation rejection. Accordingly, we investigated the presence of Treg cells and their cytokines IL-10 and TGF-β, as well as their potential to suppress T-cell effector functions in AD skin. Here, we show that IL-10–secreting Tr1 cells, but not FoxP3$^+$CD4$^+$CD25$^+$ T cells, infiltrate lesional AD skin, demonstrating an imbalance in T-cell regulation in the affected organ.

METHODS

Subjects

PBMCs were isolated from peripheral blood of 15 healthy volunteers or patients with AD (aged 19-45 years) hypersensitive to house dust mite (HDM) or birch pollen allergens and then purified or cultured to provide the various types of T cells used in this study.

Twenty-four–hour positive atopy patch test (APT) biopsies were taken of 3 patients with AD at the University Medical Center Utrecht, The Netherlands, as previously described.[15] Three psoriasis biopsies were obtained from the ZAUM-Center for Allergy and Environment, Munich, Germany. Lesional skin biopsies were obtained from 3 patients with allergic contact dermatitis and 8 patients with AD diagnosed according to standard criteria[16] at the allergy unit of the department of dermatology, University of Zurich. Patients with AD and nonallergic form of dermatitis (NAD)[17] were included in the study. They did not receive any systemic therapy for at least 2 weeks before taking the biopsy. All studies were approved by the ethical commissions of the Canton of Graubunden, Switzerland, the Zurich University, Switzerland, the ZAUM, Munich, Germany, or the University Medical Center Utrecht, The Netherlands.

Purification and *in vitro* differentiation of T-cell subsets

Cytokine-secreting cells. PBMCs were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood. Cells were washed 3 times and resuspended in RPMI 1640 medium supplemented as described.[12] Cells, 2.5 × 10^7, were stimulated with 0.3 μmol/L Der p 1 in 5 mL medium in 6-well plates (Corning-Costar Corp, Cambridge, United Kingdom). Secreting T cells were purified by immunomagnetic separation using the cytokine-secretion assay (AutoMacs; Miltenyi Biotec, Bergish Gladbach, Germany) for IL-4, IL-10, or IFN-γ as previously described.[12] Purified IL-4–secreting cells, IL-10–secreting cells, and IFN-γ–secreting cells were stimulated in complete culture medium with 1 nmol/L doses of growth factors: IL-2 (Roche, Basel, Switzerland) and IL-4 (Novartis, Basel, Switzerland), IL-2 and IL-15 (Peprotech, London, United Kingdom), and IL-2, respectively, and the following combination of mAbs to T-cell surface molecules: anti-CD2 (4B2 and 6G4, each 0.5 μg/mL), anti-CD3 (0.5 μg/mL), and anti-CD28 (0.5 μg/mL; all from CLB, Amsterdam, The Netherlands). Their cytokine profiles have been previously reported as T_H2-like, Tr1-like, and T_H1-like cells, respectively.[12]

CD4$^+$CD25$^+$ T cells. CD4$^+$CD25$^+$ T cells were purified from PBMCs of healthy donors by using the CD4$^+$CD25$^+$ regulatory T-cell isolation reagents (Miltenyi Biotec).[18] The expression of FoxP3 on CD4$^+$CD25$^+$ T cells was significantly higher than on CD4$^+$CD25$^-$ T cells, as previously reported.[19] Purified CD4$^+$CD25$^+$ T cells were cultured for 18 hours in complete RPMI 1640 medium containing IL-2 before being used in experiments.

T_H1 cells. CD45RA$^+$ T cells were purified by depletion of PBMCs using Pan T-cell isolation reagents (Miltenyi Biotec) and anti-CD45RO labeled microbeads (Miltenyi Biotec). Naive T cells (1 × 10^5 cells in 500 μL complete RPMI 1640, in 48-well plates; Corning-Costar Corp) were cultured with IL-2 (50 ng/mL), IL-12 (25 ng/mL), anti-CD2/3/28 mAb, and anti–IL-4 mAb (10 μg/mL) for 12 days to generate T_H1 cells.[20]

AD skin–derived T cells. T cells from the epidermis of lesional biopsies of patients with AD were isolated as previously described.[5] FoxP3 mRNA expression was analyzed as previously described.[19]

Keratinocyte cultures

Primary human keratinocytes (pooled normal human epidermal keratinocytes from neonatal skin) were purchased from BioWhittaker, Verviers, Belgium; PromoCell GmbH, Heidelberg, Germany; or Invitrogen, Basel, Switzerland, and grown in fully supplemented keratinocyte growth medium (KGM-2 bullet kit, BioWhittaker). During experiments, hydrocortisone was left out of the medium. Immortalized human HaCaT keratinocytes (a gift from Prof Dr N. E. Fusenig, Heidelberg, Germany) were grown in complete RPMI 1640 medium.

T-cell–keratinocyte cocultures

Keratinocytes were first seeded into 48-well or 96-well plates (Corning-Costar Corp) and were incubated to allow attachment and formation of a 75% to 90% confluent monolayer. After refreshing the medium, cytokines IFN-γ (Peprotech), soluble Fas ligand (Alexis Corp, Lausen, Switzerland), TNF-α (Alexis Corp; all 10 ng/mL), IL-10 (20 ng/mL; Peprotech), and TGF-β (2 ng/mL; R&D Systems Inc, Abingdon, United Kingdom) were used in different combinations. IL-10 and TGF-β were added at least 2 hours before if combined with other cells and/or cytokines. In cocultures with keratinocytes, T cells were incubated for only 24 hours and then washed away. If effector and IL-10–secreting Tr1 or CD4$^+$CD25$^+$ T cells were combined, regulatory cells were added at least 2 hours earlier. Of each type of T cell, 2.5 to 5 × 10^4 (96-well or 48-well plate, respectively) were used per well.

Artificial skin equivalents

Skin equivalents were cultured on dead de-epidermized dermis from human foreskin. A total of 4 × 10^5 third passage primary human keratinocytes were seeded onto the de-epidermized dermis and grown in an air-fluid interface for 10 days in modified Greens medium.[21] Fully differentiated artificial skin equivalents were grown for 4 days in a transwell system, while combinations of IFN-γ, soluble Fas ligand (sFasL), IL-10, and TGF-β were added directly to the medium below the insert. After 4 days, the skin pieces were snap-frozen in liquid nitrogen and embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands).

Viability and apoptosis detection

Keratinocyte viability was evaluated by means of ethidium bromide (25 μmol/L; Sigma Chemical Co, St Louis, Mo) exclusion and flow cytometry (EPICS XL-MCL flow cytometer; Coulter Corp, Hialeah, Fla). Hoechst staining was performed according to Norris et al[22] on cytospin samples and frozen sections of artificial skin equivalents. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining was performed on frozen sections of artificial skin equivalent by using the MEBSTAIN apoptosis kit II (MBL, Naka-ku Nagoya, Japan). Stained samples were mounted with Fluorescent Mounting Medium (DAKO, Glostrup, Denmark) and subsequently evaluated under an ultraviolet microscope (Axiovert 405M; Carl Zeiss AG, Feldbach, Switzerland).

Immunohistochemistry

Biopsy specimens were taken from lesions 3 to 6 days old and positive 24-hour APT of patients with AD, and from normal skin

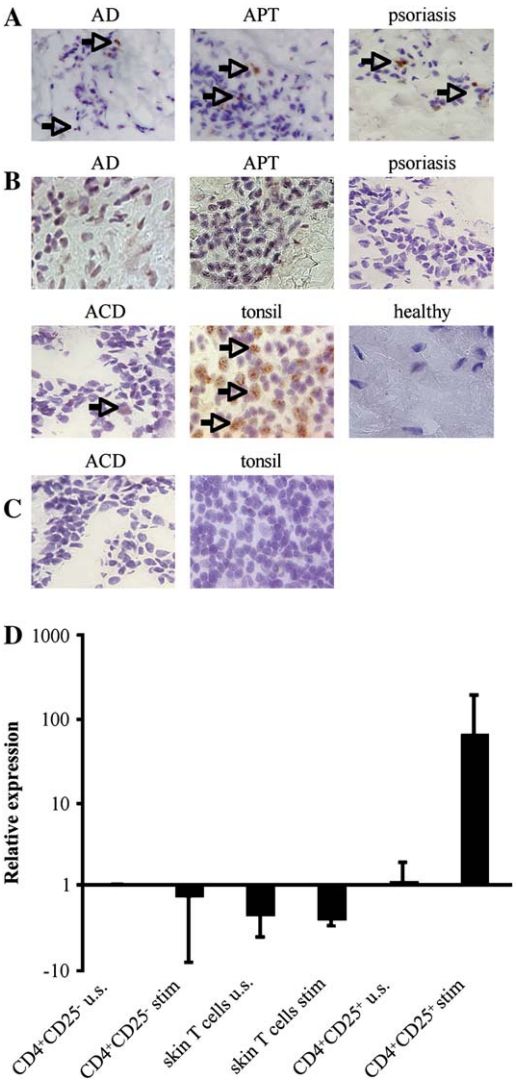

FIG 1. A, CD25 expression in the dermis of AD, APT, and psoriasis skin. **B,** FoxP3 staining in corresponding skin diseases (*ACD,* allergic contact dermatitis) and human tonsil. *Arrows* indicate positive cells. **C,** Blockage of FoxP3 staining with specific peptide. **D,** FoxP3 mRNA expression in skin T cells and blood $CD4^+CD25^+$ Treg cells relative to $CD4^+CD25^-$ T cells (*u.s.,* unstimulated; *stim,* anti-CD2, anti-CD3, and anti-CD28 mAbs stimulation for 2 hour). Same results obtained in ≥3 samples.

of healthy individuals. Frozen sections were stained by using the ready-to-use Vectastain Universal Elite Kit (Vector Laboratories, Burlingame, Calif). Primary antibodies were antihuman IL-10, antihuman TGFβ1 (both R&D Systems Inc), antihuman IL-10R1, antihuman TGFβRI, anti-human TGFβRII (all Santa Cruz Biotechnology Inc, Santa Cruz, Calif), antihuman FoxP3 ab10563 (Abcam, Cambridge, United Kingdom), antihuman CD25 (BD Biosciences Pharmingen, San Diego, Calif) and polyclonal rabbit or mouse IgG (Santa Cruz Biotechnology) as isotype controls. FoxP3 peptide ab14151 (0.125 μg/mL; Abcam) was used as a control to block anti-FoxP3 binding.

Statistical analysis

All data are expressed as means ± SDs. Statistical analysis was performed by using the Student *t* test and Mann Whitney *U* test for samples n < 6 (*$P < .05$).

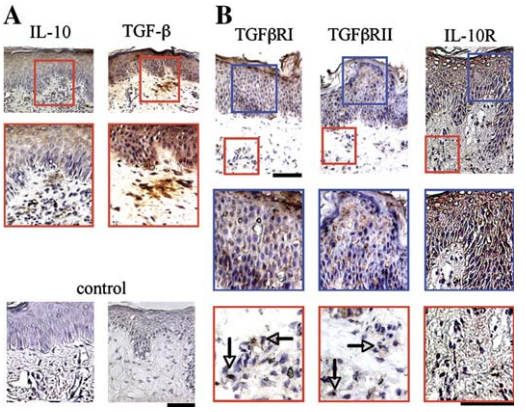

FIG 2. A, IL-10 and TGF-β expression in AD skin. **B,** TGFRI and TGFRII were expressed throughout the skin, IL-10R almost exclusively in the epidermis. *Arrows* indicate positive cells. *Highlighted* areas magnified below. Scale bar: 50 μm. Similar results obtained in ≥3 patients.

RESULTS

Expression of IL-10 and TGF-β as well as their receptors, but not FoxP3, in lesional AD skin

To investigate the expression and function of Treg cells in AD, we looked at whether the Tr1 cell-specific cytokines, IL-10 and TGF-β, or $CD4^+CD25^+$ Treg cell-specific transcription factor FoxP3 are expressed in lesional AD skin. Despite the presence of large numbers of $CD25^+$ cells (Fig 1, *A*), we did not detect any $FoxP3^+$ cells, neither in the dermal infiltrate of chronic lesional AD skin nor in acutely inflamed skin 24 hours after APT, psoriatic skin, or healthy skin (Fig 1, *B*). FoxP3 was detectable abundantly on T cells in the follicular area of the tonsil, and on approximately 1% of infiltrating T cells in allergic contact dermatitis skin by an exclusively nuclear staining. The binding of FoxP3 antibody could be blocked by preincubating the antibody with specific FoxP3 peptide (Fig 1, *C*). Analysis of mRNA levels confirmed that T cells isolated from AD skin express very low levels of FoxP3, comparable to $CD4^+CD25^-$ T cells. $CD4^+CD25^+$ Treg cells express relatively high levels of FoxP3 mRNA, which are upregulated on activation (Fig 1, *D*). Both IL-10 and TGF-β were abundantly present throughout epidermal keratinocyte layers as well as in dermal mononuclear cell infiltrate of affected skin (Fig 2, *A*). TGFβRI and TGFβRII were highly expressed in the epidermis and dermis of affected skin, whereas IL-10R was expressed in the whole epidermal layers, but only on a few cells in the dermal infiltrate (Fig 2, *B*).

IL-4–secreting, IL-10–secreting, and IFN-γ–secreting T cells are present in both AD and NAD skin lesions

Next, we isolated and characterized T cells from lesional skin biopsies of 4 patients with AD and 4 patients with NAD. Cytokine patterns determined by ELISA

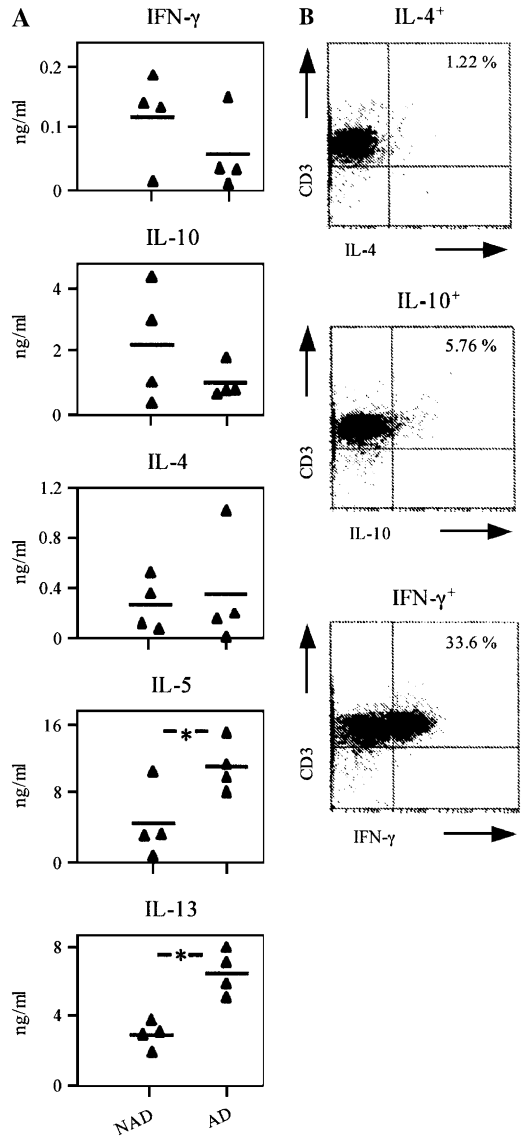

FIG 3. Cytokine profile of T cells isolated from AD skin. **A,** T cells isolated from AD and NAD skin restimulated with anti-CD2, anti-CD3, and anti-CD28 mAbs. Cytokines were measured after 72 hours in supernatants, by ELISA. *$P < .005$). **B,** Skin T cells were stimulated for 48 hours with anti-CD2, anti-CD3, and anti-CD28 antibodies. IL-4–secreting, IL-10–secreting, and IFN-γ–secreting $CD3^+$ T cells were demonstrated.

showed a significant increase in IL-5 and IL-13 in AD. IL-10, IFN-γ, and IL-4 secretion did not differ significantly between the 2 types of AD (Fig 3, *A*). In either type of AD, both IFN-γ as an effector cytokine and IL-10 as a regulatory cytokine were detectable. A quantitative determination of IL-4–secreting, IL-10–secreting, and IFN-γ–secreting T cells was possible by capturing the secreted cytokine on the surface of the T cell. The majority of T cells isolated from AD skin were IFN-γ–secreting cells. A considerable percentage of IL-10–secreting cells, but only a minor fraction of IL-4–secreting cells, were found among skin T cells (Fig 3, *B*).

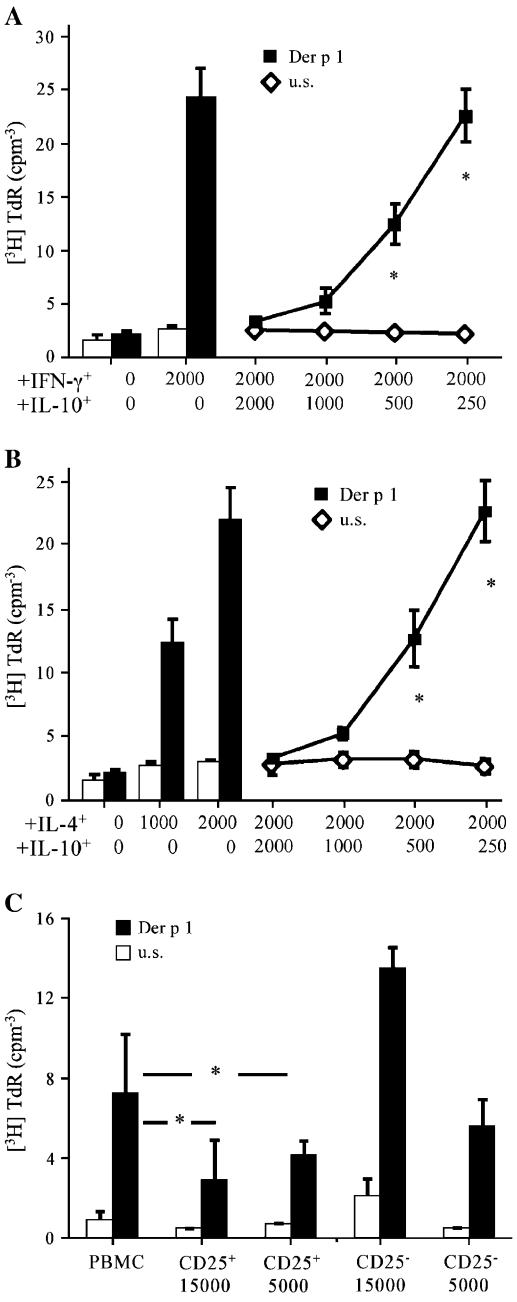

FIG 4. Tr1 and $CD4^+CD25^+$ T cells suppress allergen-specific T cells. Der p 1–specific IFN-γ–secreting, IL-4–secreting, and IL-10–secreting T cells were purified from peripheral blood. IFN-γ–secreting **(A)** and IL-4–secreting **(B)** T cells were added to PBMCs and stimulated with Der p 1 in the absence or presence of IL-10–secreting Tr1 cells. One experiment representative of 8 is shown. **C,** $CD4^+CD25^+$ T cells suppress the Der p 1–induced proliferation of PBMCs of donors allergic to HDM (n = 3). *u.s.,* Unstimulated. [^{3}H] Thymidine (TdR) incorporation determined after 5 days. *$P < .05$.

Allergen-specific IL-10–secreting Tr1 cells and $CD4^+CD25^+$ Treg cells inhibit activation of allergen-specific effector T cells

To establish the immune regulatory capacity of the IL-10–secreting Tr1-like cells used in this study, we

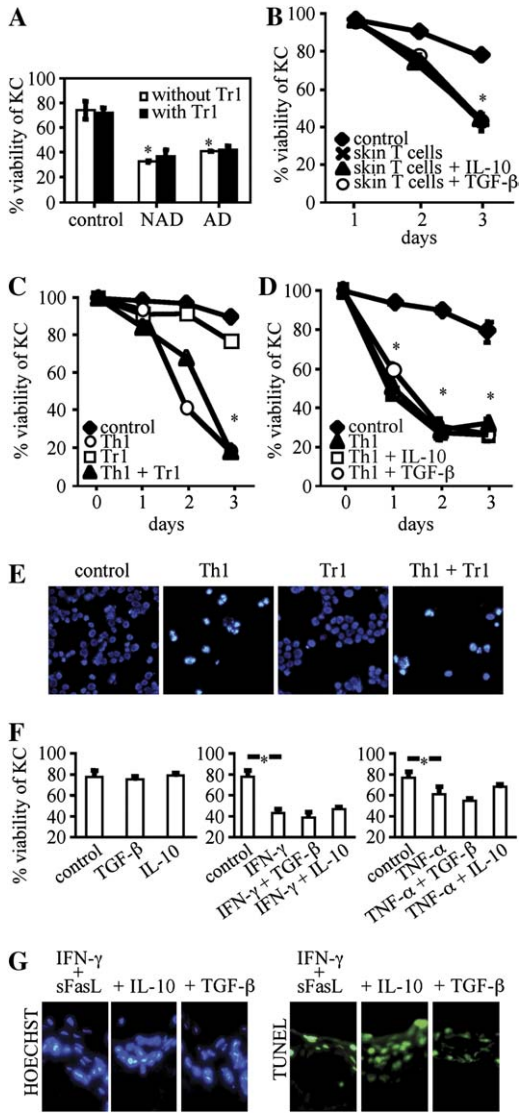

FIG 5. IL-10, TGF-β, and Tr1 cells do not influence keratinocyte (KC) apoptosis. **A,** Viability of KCs 3 days after coculture with T cells isolated from AD or NAD skin. IL-10–secreting Tr1 cells were added in equal amounts to skin T cells. **B,** IL-10 and TGF-β do not influence skin T-cell–induced KC death. **C,** KCs cocultured with Tr1 cells and T_H1 cells. **D,** IL-10 and TGF-β do not influence T_H1 cell–induced KC death. **E,** Staining of KCs 3 days after coculture with T_H1 cells and/or IL-10–secreting Tr1 cells with Hoechst 33342. **F,** KCs stimulated for 3 days with IFN-γ, TNF-α, IL-10, and TGF-β. *(A-F)* One experiment representative of 3 is shown. Viability determined by ethidium bromide exclusion. **G,** Artificial skin equivalents were cultured with IFN-γ and sFasL for 4 days, in the presence or absence of IL-10 (10 ng/mL) and TGF-β (2 ng/mL). $*P < .05$. *TUNEL,* Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

examined their effect on the antigen-specific activation of IL-4–secreting T_H2-like and IFN-γ–secreting T_H1-like T cells. Freshly purified T_H cells selected for their specificity against the HDM allergen Der p 1 showed increased proliferation upon encounter with this allergen when added to autologous PBMCs (Fig 4, *A and B*). An equal amount of IL-10–secreting Tr1 cells to that of IL-4–

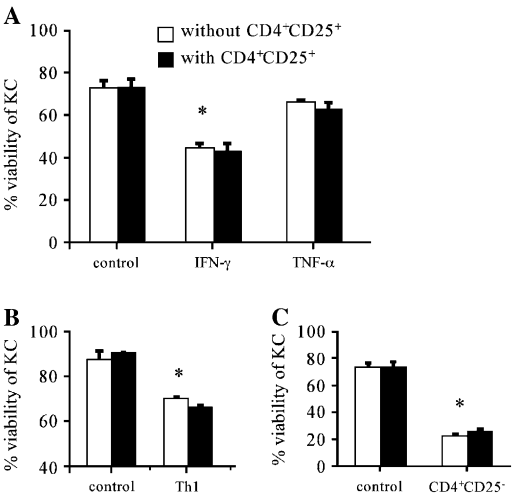

FIG 6. $CD4^+CD25^+$ Treg cells do not affect keratinocyte (KC) death induced by **(A)** IFN-γ and sFasL (72 hours), **(B)** *in vitro*–differentiated T_H1 cells (48 hours), or **(C)** $CD4^+CD25^-$ T cells, preactivated for 48 hours. Viability was measured by ethidium bromide exclusion 48 to 72 hours after coculture. Experiments were performed at least twice in triplicate cultures. $*P < .05$.

secreting or IFN-γ–secreting cells nearly abolished this response. This shows that IL-10–secreting Tr1 cells can suppress allergen-specific activation of both T_H2-like and T_H1-like cells. Similarly, $CD4^+CD25^+$ T cells showed significant suppression of Der p 1–specific proliferation of PBMCs from donors allergic to HDM (Fig 4, *C*).

Tr1 cells, their cytokines IL-10 and TGF-β, and $CD4^+CD25^+$ Treg cells do not suppress T-cell–induced keratinocyte apoptosis

T-cell–induced keratinocyte apoptosis plays an essential role in the development of eczematous lesions in AD. Accordingly, we investigated whether Tr1 cells or their suppressive cytokines IL-10 and TGF-β can suppress skin T-cell–induced, *in vitro* differentiated T_H1 cell–induced, or IFN-γ and sFasL–induced keratinocyte apoptosis in cocultures and artificial skin equivalents. Keratinocyte death induced by skin T cells did not show any difference between T cells isolated from AD or NAD biopsies. In both cases, keratinocyte death induced by preactivated skin T cells was not prevented by the addition of IL-10–secreting Tr1 cells in coculture (Fig 5, *A*). Moreover, skin T-cell–induced keratinocyte death was not blocked by the addition of IL-10 or TGF-β (Fig 5, *B*).

The suppressive capacity of IL-10–secreting Tr1 cells on IL-12–driven T_H1 cell–induced keratinocyte apoptosis was further analyzed in cocultures with human keratinocytes. IL-10–secreting Tr1 cells did not affect keratinocyte viability alone, nor did they suppress T_H1 cell–induced keratinocyte death in cocultures (Fig 5, *C*). Addition of IL-10 or TGF-β to keratinocytes during coculture again did not suppress T_H1-induced keratinocyte death (Fig 5, *D*). Bright, condensed, and fragmented staining of keratinocyte nuclei with Hoechst 33342 dye, 3 days after coculture with T_H1 cells, further confirmed these findings

and suggested that cell death was in the form of apoptosis, which was not inhibited by IL-10–secreting Tr1 cells (Fig 5, *E*).

Because direct T-cell–keratinocyte contact is not essential for the pathology of AD, we performed the same experiments based solely on cytokines. Keratinocytes were cultured with the effector cytokines IFN-γ and TNF-α. Again, a substantial reduction in the viability of keratinocytes was observed, mainly after culture with IFN-γ and to a lesser extent with TNF-α after 3 days. As in the experiments with T_H1 cells, the induced keratinocyte death was not suppressed by IL-10 and TGF-β (Fig 5, *F*). Similar results were obtained with HaCaT and primary human keratinocytes.

The differentiation status of keratinocytes was hypothesized to play a role in the observations with monolayer cell cultures. Accordingly, the effects of the aforementioned cytokines were studied in a model of artificial skin equivalents in a 3-dimensional structure that involves primary human keratinocytes, dermal fibrocytes, and extracellular matrix proteins. IFN-γ and sFasL induced severe cell death throughout the epidermis of skin equivalents after 4 days (Fig 5, *G*). Similar to monolayer keratinocyte cultures, IL-10 or TGF-β did not suppress the apoptosis of keratinocytes in artificial skin equivalents.

A direct interaction of $CD4^+CD25^+$ Treg cells and keratinocytes was investigated by the addition of $CD4^+CD25^+$ Treg cells to TNF-α–stimulated or IFN-γ–stimulated keratinocytes. The reduced viability of keratinocytes observed after 3 days was not affected by $CD4^+CD25^+$ Treg cells, similar to the findings with IL-10–secreting Tr1 cells (Fig 6, *A*). Like Tr1 cells, $CD4^+CD25^+$ Treg cells did not induce keratinocyte apoptosis. To assess whether $CD4^+CD25^+$ Treg cells suppress T_H1-induced or $CD4^+CD25^-$ T-cell–induced keratinocyte apoptosis, $CD4^+CD25^+$ Treg cells were added to a coculture of T_H1 or $CD4^+CD25^-$ T cells with keratinocytes. In both cases, $CD4^+CD25^+$ Treg cells did not inhibit keratinocyte death (Fig 6, *B and C*).

DISCUSSION

During the last decade, a significant amount of data has accumulated on the suppressive effects of Tr1 or $CD4^+CD25^+$ T-regulatory cells in models of autoimmunity, allergy, transplantation tolerance, tumor tolerance, and chronic infections.[12,23] The efficacy of various Treg cell subsets in the suppression of inflammation has tempted scientists to speculate that increasing Treg cell numbers may suppress inflammation and tissue injury in affected organs. In the current study, we show that $FoxP3^+CD25^+$ T cells are not present in AD skin, whereas Tr1 cells, their suppressive cytokines IL-10 and TGF-β, and receptors for these cytokines are abundantly expressed. Both $CD4^+CD25^+$ T regulatory cells and Tr1 cells can efficiently suppress activation of T_H1 and T_H2 cells stimulated with allergen/antigen. However, the effector function of preactivated T cells, namely keratinocyte apoptosis, is not affected either by $CD4^+CD25^+$ T cells or Tr1 cells and their suppressive cytokines IL-10 and TGF-β.

One difficulty in comparing the results of different studies with Treg cells is the variation in regulatory cell types. The IL-10–secreting Tr1 cells used in this study were selected for their allergen-induced IL-10 secretion as previously described.[12] $CD4^+CD25^+$ T cells were isolated from peripheral blood of healthy and donors allergic to HDM. Most studies with IL-10–secreting Tr1 cells and $CD4^+CD25^+$ T cells have focused on their ability to inhibit proliferation of responder cells. Here, we demonstrate that IL-10–secreting Tr1 cells can inhibit the allergen-specific proliferation of IL-4–secreting T_H2 as well as of IFN-γ–secreting T_H1 cells. In addition, a considerable percentage of T cells isolated from lesional AD skin are IL-10–secreting cells. Furthermore, we showed the presence of Tr1 cytokines, IL-10 and TGF-β, and their receptors in biopsies of AD skin. Supporting these findings, overexpression of IL-10 was previously described in AD,[24] and all isoforms of TGF-β have been described to be expressed in nonaffected skin, with an upregulation during wound repair.[25]

Previously, $CD4^+CD25^+$ Treg cells and $CLA^+CD4^+CD25^+$ T cells have been demonstrated to be elevated in peripheral blood of patients with AD compared with healthy controls or patients with asthma.[26] Although we found a high amount of $CD25^+$ cells in the dermal infiltrate of AD skin, we did not detect any FoxP3 expression. This shows that these $CD25^+$ cells present in the skin are activated T cells and not regulatory $CD4^+CD25^+$ T cells. Similarly, circulating CLA^+CD4^+ or $CD8^+$ T cells have been demonstrated to express CD25 highly and display effector functions by inducing IgE production by B cells and prolonged survival of eosinophils.[26] Mutations in the FoxP3 gene have previously been reported to play a critical role in the onset of immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome, an X-linked recessive immunological disorder. This rare disease is often associated with eczema (4 out of 5 patients in 1 study) and high levels of IgE.[27] Together, these data suggest that there might be an essential role for $CD4^+CD25^+FoxP3^+$ Treg cells in controlling inflammation of the skin, a system apparently malfunctioning in AD.

Both IL-10R and TGF-βR have been described in the epidermis of healthy skin, with a marked upregulation of the latter during wound repair.[28,29] In contrast to psoriasis,[28] IL-10R was abundantly expressed throughout affected epidermis in AD. Although no effect of IL-10 on keratinocytes has previously been reported, this expression of IL-10R suggests that there might be a role for IL-10 in the control of keratinocyte death in AD. Accordingly, we examined the role of IL-10–secreting Tr1 and $CD4^+CD25^+$ Treg cells in the control of keratinocyte apoptosis on both a cell-to-cell contact and a cytokine-mediated level. We found that T cells isolated from lesional skin of patients with AD induced apoptosis of keratinocytes, despite the presence of a considerable amount

of IL-10–secreting Tr1 cells. The addition of exogenous IL-10–secreting Tr1 cells isolated from peripheral blood of healthy donors did not prevent the apoptosis of keratinocytes in these cocultures. A defect in regulation has been suggested as a determinant in the ongoing effector functions of AD skin T cells. Superantigens, present in the skin of more than 90% of patients with AD, can induce strong proliferation of $CD4^+$ and $CD8^+$ T cells in the skin.[30] Strong binding of superantigens to the TCR in conjunction with CD28 costimulation[31] was shown to render T cells insensitive to suppression by $CD4^+CD25^+$ Treg cells[32] and IL-10.[33] Superantigens also abrogate immune suppression by corticosteroids,[34] which operate via the induction of Treg cells[19] This provides a possible explanation for the absence of inhibition of skin T-cell–induced keratinocyte apoptosis by Tr1 or $CD4^+CD25^+$ Treg cells. To exclude this phenomenon, the same experiments were repeated with Th1 cells and $CD4^+CD25^-$ T cells from healthy donors. The apoptosis induction by these cell types was comparable to that seen with skin T cells, but Tr1 and $CD4^+CD25^+$ Treg cells did not induce keratinocyte apoptosis. Because an inhibitory effect might be cell contact–independent and cytokine levels secreted by T cells may vary, similar experiments were performed with effector cytokines such as TNF-α and IFN-γ and suppressor cytokines like IL-10 and TGF-β. Again, no inhibitory effect of IL-10 and TGF-β was seen on IFN-γ–induced or TNF-α–induced apoptosis.

In perspective, 4 distinct stages play an important role in allergic inflammation of the skin. The first is the activation of T cells by allergens or superantigens, followed by organ-selective homing, whereby cells are influenced by the network of chemokines in the skin.[2,35] The third stage is classified by prolonged survival of inflammatory cells within the inflamed skin and reactivation, by allergens and/or superantigens. Finally, the effector role of T cells in the skin is characterized by the induction of keratinocyte apoptosis and development of spongiosis, all of which are important factors in AD. Thus, regulatory T cells of either Tr1 or $CD4^+CD25^+$ Treg phenotype can suppress antigen-specific activation of T cells (stage 1 and stage 3), but they cannot prevent activated effector T-cell–induced keratinocyte apoptosis (stage 4). In addition, taken together with AD and hyper-IgE in the phenotype of immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome, the absent expression of $CD4^+CD25^+FoxP3^+$ Treg cells in AD and psoriasis skin suggests a dysregulated control of inflammation, particularly by natural Treg cells.

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6.2 Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells.

J exp Med., 2004. 199: 1567-1575

Immune Responses in Healthy and Allergic Individuals Are Characterized by a Fine Balance between Allergen-specific T Regulatory 1 and T Helper 2 Cells

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Abstract

The mechanisms by which immune responses to nonpathogenic environmental antigens lead to either allergy or nonharmful immunity are unknown. Single allergen-specific T cells constitute a very small fraction of the whole $CD4^+$ T cell repertoire and can be isolated from the peripheral blood of humans according to their cytokine profile. Freshly purified interferon-γ–, interleukin (IL)-4–, and IL-10–producing allergen-specific $CD4^+$ T cells display characteristics of T helper cell (Th)1-, Th2-, and T regulatory (Tr)1–like cells, respectively. Tr1 cells consistently represent the dominant subset specific for common environmental allergens in healthy individuals; in contrast, there is a high frequency of allergen-specific IL-4–secreting T cells in allergic individuals. Tr1 cells use multiple suppressive mechanisms, IL-10 and TGF-β as secreted cytokines, and cytotoxic T lymphocyte antigen 4 and programmed death 1 as surface molecules. Healthy and allergic individuals exhibit all three allergen-specific subsets in different proportions, indicating that a change in the dominant subset may lead to allergy development or recovery. Accordingly, blocking the suppressor activity of Tr1 cells or increasing Th2 cell frequency enhances allergen-specific Th2 cell activation ex vivo. These results indicate that the balance between allergen-specific Tr1 cells and Th2 cells may be decisive in the development of allergy.

Key words: peripheral tolerance • allergens • suppression • interleukins • immune regulation

Introduction

The immune system must distinguish between innocuous and pathological antigens to prevent unnecessary and self-destructive immune responses (1, 2). A central finding from experimental models and human studies shows that allergic diseases are due to an aberrant immune response mediated through a key effector cell, the Th type 2 cell and an associated cytokine pattern including IL-4, IL-5, and IL-13 (3, 4). Therefore, the most pronounced findings with potential relevance to allergy therapy are related directly to the control of these Th2 immune effectors. There is strong evidence that peripheral T cell regulation plays a crucial role in the control of harmful T cell responses. To avoid chronic cell activation and inflammation against nonpathogenic antigens through ingestion and inhalation, the immune system has developed efficient peripheral tolerance mechanisms. Since the early 1970s (5), different subtypes of regulatory and suppressor cells and mechanisms that may play a role in peripheral tolerance have been demonstrated, and their biology has been the subject of intensive investigation (6–14).

The online version of this article contains supplemental material.

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Abbreviations used in this paper: CTLA-4, CTL antigen 4; EF-1α, elongation factor-1α; L, ligand; PD-1, programmed death 1; PPD, purified protein derivative of *Mycobacterium bovis*; Tr, T regulatory.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2004/06/1567/9 $8.00
Volume 199, Number 11, June 7, 2004 1567–1575
http://www.jem.org/cgi/doi/10.1084/jem.20032058

A great deal of uncertainty remains about differentiation factors, antigen specificity, and mechanisms of action of T regulatory (Tr) cells. Several types of Tr cells have been described with a unique mechanism of action that varies depending on the experimental model. Recent studies have shown that Tr cells act as suppressor T cells that down-regulate other effector T cells and inflammation models in chronic infection, organ transplantation, and autoimmunity (10, 15–17). Studies on the immune response to allergens provide well-defined models for understanding the regulation and circumvention of antigen-specific T cell responses. The symptoms of IgE-mediated allergy rhinitis, conjunctivitis, and asthma can be ameliorated by the temporary suppression of mediators and immune cells (such as antihistamines and corticosteroids; reference 18). However, the only long-term solution for the treatment of allergy is allergen-specific immunotherapy by the administration of high doses of allergen or allergen peptides that specifically target T cells over a long period of time (19, 20). Successful venom and aeroallergen immunotherapy was found to be associated with the induction of peripheral tolerance in T cells by generation of Tr cells that secrete the suppressive cytokines IL-10 and TGF-β, suggesting that generation of Tr1 cells might play a role in healthy immune response (11, 21).

Studies on immune response to allergens in healthy individuals demonstrated that a peripheral T cell repertoire to allergens exists that recognizes the same T cell epitopes as allergic patients (22, 23). In the present work, we used a direct approach by purification, characterization, and frequency determination of allergen-activated T cells according to their cytokine secretion profile. This enabled a suitable human model to investigate how harmless environmental proteins are recognized and tolerated by the immune system. We showed that healthy and allergic immune response to common environmental proteins is characterized by a delicate balance in frequency of allergen-specific Tr1 cells and allergen-specific Th2 cells.

Materials and Methods

Study Population. Heparinized peripheral blood samples from 31 healthy individuals (mean age, 36 yr) with no history of atopy and 8 birch pollen and 9 house dust mite allergic patients, who were diagnosed by positive skin test reactivity and high specific IgE antibodies (>0.70 kU/l, Pharmacia Cap assay) were studied. Total IgE levels of healthy individuals were <88 U/ml, and allergic individuals were between 92 and 298 U/ml. The study was approved by the ethical commission of Davos, Switzerland.

Antigens. rBet v 1 of birch pollen (*Betula verrucosa*), rDer p 1 of house dust mite (*Dermatophagoides pteronyssinus*), rPyr c 5 of pear (*Pyrus communis*), and rCor a 1 of hazelnut (*Corylus avellana*) were used. rPyr c 5 and r Cor a 1 were provided by S. Vieths and D. Lüttkopf (Paul Ehrlich Institute, Langen, Germany). All of the allergens did not contain detectable amounts of LPS and were >99% pure. Purified protein derivative of *Mycobacterium bovis* (PPD) was obtained from the Serum Institute, Copenhagen, Denmark. Tetanus toxoid was obtained from the Institute Berne, Switzerland.

Purification of Allergen-specific IL-4–, IFN-γ–, and IL-10–secreting Cells. PBMCs were isolated by Ficoll (Biochrom) density gradient centrifugation of peripheral venous blood, and cells were washed three times and resuspended in RPMI 1640 medium supplemented as described previously (8). 2.5×10^7 cells were stimulated with 0.3 μM antigens in 5 ml of medium in six-well plates in duplicates (Costar Corp.). After 12 h of stimulation in humidified 5% CO_2, cells were harvested and labeled with 50 μg/ml anti–IFN-γ/CD45, anti–IL-4/CD45, or anti–IL-10/CD45 Ab–Ab conjugates (Miltenyi Biotec) for 10 min at a concentration of 10^8 cells/ml in ice-cold RPMI 1640 medium (24). The cells were diluted with 37°C warm medium to a final concentration of 10^6 cells/ml and allowed to secrete and capture the respective cytokines for 45 min at 37°C. After capturing the secreted cytokines on their surface, cells were centrifuged at 300 *g* for 5 min at 4°C and resuspended at a concentration of 10^8 cells/ml in ice-cold buffer containing 0.5% BSA and 5 mM EDTA (both obtained from Sigma-Aldrich) in PBS. The cells were stained with 5 μg/ml PE-conjugated anti–IFN-γ, anti–IL-10, or anti–IL-4 for 10 min at 4°C. The cells were washed and resuspended in 400 μl BSA-EDTA PBS and magnetically labeled for 15 min at 4°C with 100 μl of anti-PE microbeads. After washing, labeled cells were purified by immunomagnetic separation (AutoMacs; Miltenyi Biotec). The cells were counterstained by FITC-labeled anti-CD4 and anti-CD8 mAb (Immunotech) and analyzed in a flow cytometer (Epics XL; Beckman Coulter; Fig. S1). The purity of allergen-specific $CD4^+$ cytokine-secreting cells was between 88 and 96%. The frequency of allergen-stimulated and unstimulated cells was calculated by dividing the number of purified cytokine-secreting $CD4^+$ T cells by the initial number of $CD4^+$ T cells. To obtain the frequency of allergen-specific, cytokine-secreting T cells, the unstimulated cytokine-secreting $CD4^+$ T cell frequency was subtracted from allergen-stimulated cell number.

T Cell Cultures. The purified allergen-specific, cytokine-secreting T cells were used immediately or expanded in the presence of 1 nM doses of growth factors (IL-2 for IFN-γ–secreting T cells; IL-2 and IL-4 for IL-4-secreting T cells; and IL-2 and IL-15 for IL-10–secreting T cells; Novartis AG). Their cytokine profile was determined both immediately after purification and after in vitro expansion. Expanded IL-4–, IL-10–, and IFN-γ–producing cells were washed, and 5×10^5 cells were restimulated with a combination of mAbs to CD2, CD3, and CD28 molecules (each 0.5 μg/ml; CLB) in 500 μl supplemented RPMI 1640 medium in 48-well plates for 3 d in triplicates. Supernatants were harvested, and cytokines were determined by solid phase sandwich ELISAs for IFN-γ, IL-4, IL-5, IL-10, IL-13, and TGF-β as described previously (8, 21). Anti–IL-4 and anti–IFN-γ mAbs were provided by C.H. Heusser (Novartis Pharmaceuticals, Basel, Switzerland).

Allergen-specific T cell proliferative response was determined by stimulation of 2×10^5 PBMCs alone or together with expanded or freshly purified allergen-specific, cytokine-secreting T cells for 5 d with 0.3 μM of antigens in 200 μl of medium in 96-well flat-bottom tissue culture plates in triplicates (25). Autologous 3,000 rad irradiated PBMCs were used as APCs. Cells were pulsed with 1 μCi/well [^{3}H]thymidine (Dupont and NEN Life Science Products), and incorporation of labeled nucleotide was determined after 8 h in an LKB β plate reader (Wallac and Amersham Biosciences). For polyclonal activation of T cells, plates were coated for 2 h at 37°C with 10 μg/ml anti-CD3. IL-10 was neutralized in cultures with 4 μg/ml anti–IL-10R mAb (provided by K. Moore, DNAX Research Institute, Palo Alto, CA; reference 26). TGF-β was neutralized in cultures with 100 ng/ml

of recombinant human soluble TGF-β receptor II/Fc chimeric protein (R&D Systems; reference 21). Programmed death 1 (PD-1) activity was neutralized in cultures with 5 μg/ml anti–human PD-1 (Bioscience Insight Biotechnology Ltd.). CTL antigen 4 (CTLA-4) activity was neutralized with 5 μg/ml anti-CD152 F(ab)^2 (Ancell and Qbiogene). The neutralizing activity of these approaches was controlled in titrated doses. Rabbit IgG, rat IgG, mouse IgG1, or BSA (Beckman Coulter) served as control.

Flow Cytometry, Immunohistology, and ELISPOT Assay. 2.5×10^4 cells were stained with FITC-conjugated anti-CD25 mAb together with anti–CD4-ECD, anti–CD152-PE (CTLA-4; BD Biosciences), or anti–PD-1 (Bioscience Insight Biotechnology Ltd.) for 30 min at 4°C. Stained cells were fixed in 2% paraformaldehyde. The controls were FITC, PE, or ECD-conjugated mouse IgG1. For analysis of IL-10R, 5×10^4 cells were stained with 50 μg/ml of anti–IL-10α mAb (DNAX Research Institute) for 30 min and washed with 2% FCS containing PBS. FITC-conjugated anti–rat Ig was the second Ab used for 30 min.

Intracellular cytokines were detected after anti-CD2, anti-CD3, and anti-CD28 mAb stimulation for 12 h. 2 μM monensin (Sigma-Aldrich) was added during the last 10 h (11). Intracytoplasmic cytokine profile of in vitro–expanded allergen-specific T cells was determined as aforementioned. Labeled mAbs for cytokines were obtained from BD Biosciences. Immune histology was performed in cytospins of freshly purified cells by using anti–human TGF-βRI, anti–human TGF-βRII, or rabbit IgG as isotype control (all obtained from Santa Cruz Biotechnology Inc.) as described previously (27).

10^6 cells/ml PBMCs from six healthy donors were stimulated in 200 μl of medium 96-well flat bottom ELISPOT plates for 18 h (Euroclone Ltd.). Locally produced IL-4, IFN-γ, and IL-10 were captured by specific mAb. After cell lysis, trapped cytokine molecules were revealed by a secondary biotinylated detection antibody, which is in turn recognized by streptavidin conjugated to alkaline phosphatase. Colored "purple" spots developed after substrate addition were determined (ImmunoSpot; Cellular Technology Ltd.). The number of spots determined in triplicates of unstimulated wells was subtracted from 0.3 μM Der p 1–stimulated wells. 18 h was found to be the optimal time for the determination of frequency of cytokine-secreting cells, as it is the time point for highest cytokine secretion before T cell proliferation starts.

Quantitative Real-Time PCR. Immediately after purification, antigen-specific, cytokine-secreting T cells were lysed with RNeasy lysis buffer, and the RNA was isolated using the RNeasy mini kit (QIAGEN) and eluted in 30 μl ddH_2O. Reverse transcription was performed with TaqMan® reverse transcription reagents with random hexamers (Applied Biosystems). The PCR primers and probes detecting elongation factor-1α (EF-1α), IL-10, TGF-β, IL-13, and IFN-γ were designed based on sequences reported in GenBank. Primers used were as follows: EF-1α forward primer, 5′-CTGAACCATCCAGGCCAAAT-3′, EF-1α reverse primer, 5′-GCCGTGTGGCAATCCAAT3′; IL-13 forward primer A, 5′-GCCCTGGAATCCCTGATCA-3′, IL-13 reverse primer A, 5′-GCTCAGCATCCTCTGGGTCTT-3′; IFN-γ forward primer B, 5′-TCTCGGAAACGATGAAATATACAAGTTAT-3′, IFN-γ reverse primer B, 5′-GTAACAGCCAAGAGAACCCAAAA-3′; IL-10 forward primer, 5′-GGCGCTGTCATCGATTTCTT-3′, IL-10 reverse primer, 5′-TTGGAGCTTATTAAAGGCATTCTTC-3′; TGF-β1 forward primer, 5′-AAATTGAGGGCTTTCGCCTTA-3′, and TGF-β1 reverse primer, 5′-GAACCCGTTGATGTCCACTTG-3′. cDNAs were amplified using SYBR®-PCR mastermix (Applied Biosystems) according to the recommendations of the manufacturer in a total volume of 25 μl in a sequence detection system (ABI

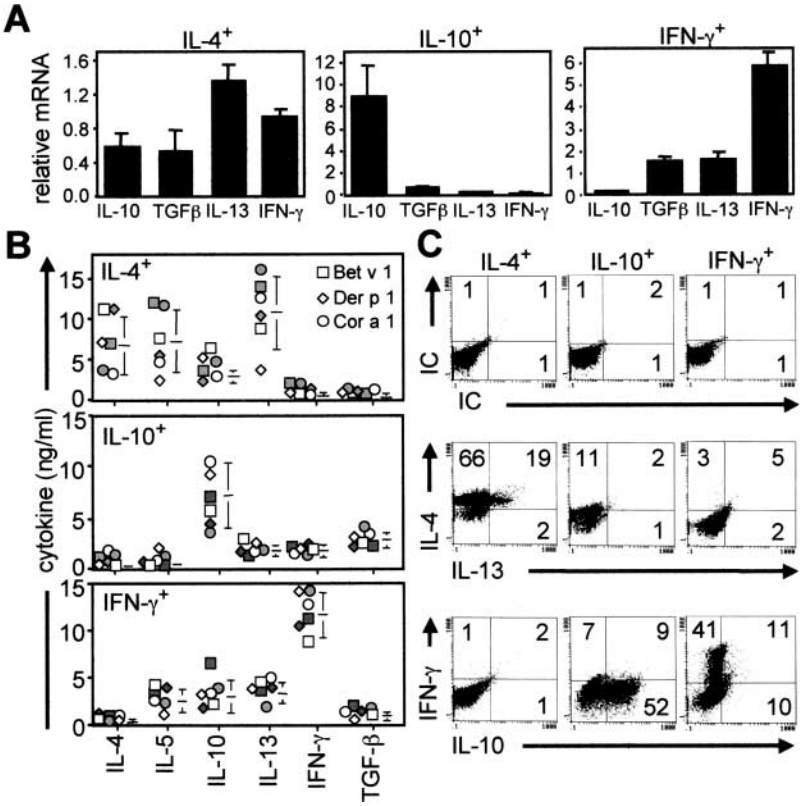

Figure 1. Allergen-specific IL-4-, IFN-γ–, and IL-10–secreting T cells represent Th2-, Th1-, and Tr1-like cells. (A) IL-10, IL-13, IFN-γ, and TGF-β mRNA were quantified by real-time PCR immediately after isolation of Der p 1– or Bet v 1–specific, cytokine-secreting T cells and their relative expression compared with the housekeeping gene EF-1α. The same results were obtained in three independent experiments. (B) Bet v 1–, Der p 1–, and Cor a 1–specific IL-4–, IFN-γ–, and IL-10–secreting T cells (one allergic and one healthy donor each; closed symbols, allergic donors; open symbols, healthy donors) were in vitro expanded for 2 wk, and their cytokine profile was determined in supernatants by ELISA 72 h after anti-CD2, anti-CD3, and anti-CD28 mAb stimulation. (C) Intracytoplasmic cytokine profile of in vitro–expanded allergen-specific T cells (the same results were obtained in 10 additional experiments). Percentage of positive cells is shown in each quadrant.

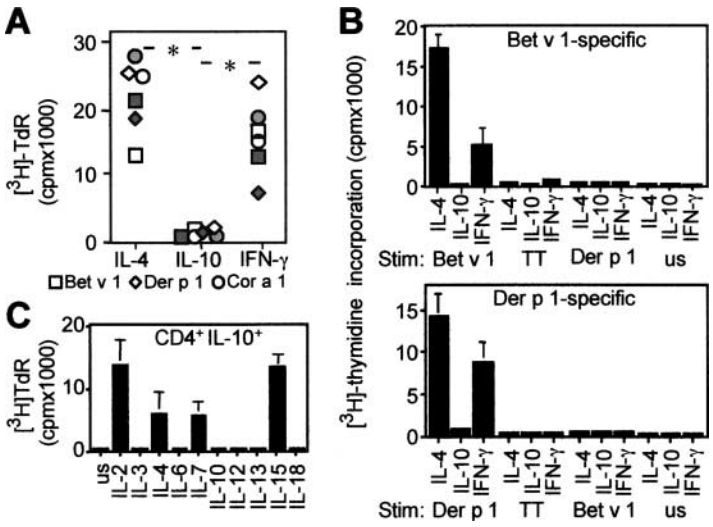

Figure 2. Specificity and growth requirements of purified allergen-specific T cells. (A) Bet v 1–, Der p 1–, and Cor a 1–specific IL-4–, IFN-γ–, and IL-10–secreting T cells (one allergic and one healthy donor each; closed symbols, allergic donors; open symbols, healthy donors) were purified and in vitro expanded, and 5×10^5 cells were cocultured with 5×10^5 autologous irradiated PBMCs as an APC source in the presence of the same antigen. [^{3}H]Thymidine incorporation (TdR) was determined after 5 d. *, $P < 0.001$. (B) Purified Bet v 1–specific and Der p 1–specific T cells did not show any proliferative response to different control antigens (two representative of six experiments are shown; mean ± SD of triplicate cultures). TT, tetanus toxoid; us, unstimulated. (C) Growth factor requirements of antigen-specific IL-10–secreting T cells. After purification, Bet v 1– or Der p 1–specific IL-10–secreting T cells were first expanded for 10 d in the presence of IL-2, washed, and cultured (5×10^4 cells) with 1-nM doses of different cytokines. [^{3}H]Thymidine incorporation was determined after 3 d (mean ± SD of three independent experiments are shown).

PRISM 7000; Applied Biosystems). Relative quantification was performed as described previously (28). All amplifications were performed in duplicates.

Statistical Interpretation. Data are expressed as mean ± SEM. Student's *t* test, Z test, and Mann-Whitney U test were used for statistical analysis.

Online Supplemental Material. Fig. S1 contains sample data of the purification and frequency calculation of allergen-specific $CD4^+$ cells according to their cytokine profile. Details of flow cytometric analysis are shown, from allergen stimulation to frequency calculation of purified IL-10–secreting Tr1 cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20032058/DC1.

Results

Allergen-specific IL-4–, IFN-γ–, and IL-10–secreting T Cells Represent Th2-, Th1-, and Tr1-like Cells. To investigate the regulation of specific immune response against environmental protein antigens, we isolated $CD4^+$ T cells specific to several food and aeroallergens from healthy and allergic individuals according to their IL-4, IFN-γ, and IL-10 secretion profile (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20032058/DC1). To confirm their cytokine profile, mRNA of IL-10, IL-13, IFN-γ, and TGF-β were quantified immediately after isolation (Fig. 1 A). Relative to the housekeeping gene EF-1α, IL-10–secreting T cells expressed significantly high IL-10 mRNA and IFN-γ–secreting T cells expressed significantly high IFN-γ mRNA. IL-13 mRNA was dominant in IL-4–secreting T cells. Quantification of cytokines after in vitro expansion of IL-4–, IL-10–, and IFN-γ–secreting T cells revealed that these subsets contain Th2-like (IL-4, IL-5, and IL-13 high), Th1-like (IFN-γ high), and Tr1-like (IL-10 and TGF-β high) cells, respectively (all $P < 0.0001$ compared with other subsets; Fig. 1, B and C). In addition, all three purified subsets consisted of some Th0 cells, which secrete both Th1 and Th2 cytokines as well as IL-10 and TGF-β. There was no difference in cytokine profile at the single T cell subset level between allergic and healthy individuals.

The antigen specificity of purified cytokine-secreting T cells was studied by stimulation with the allergen that was originally used for stimulation before purification and several control antigens in the presence of autologous APCs. Consistently, IL-10–secreting T cells showed very little or no allergen-induced proliferation (Fig. 2 A). The proliferative response of allergen-specific IL-4– and IFN-γ–secreting T cells was significantly high compared with IL-10–

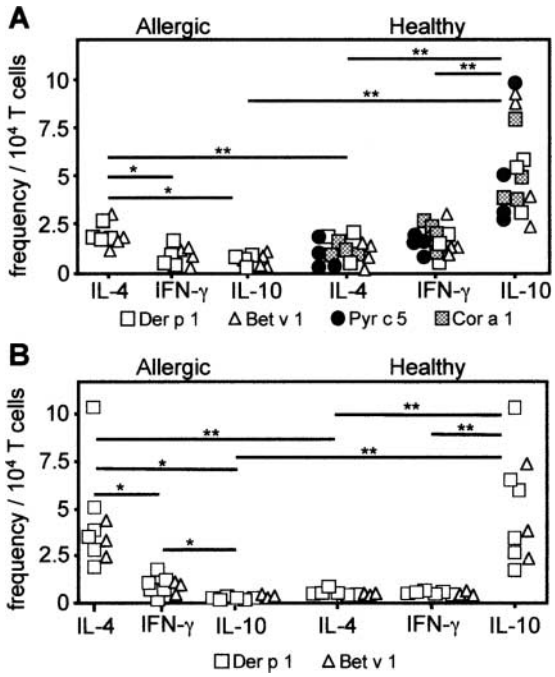

Figure 3. Increased frequency of allergen-specific Tr1 cells in healthy individuals and Th2 cells in allergic individuals. (A) Frequency of Der p 1– and Bet v 1–specific, cytokine-secreting $CD4^+$ T cells from eight allergic individuals and Cor a 1–, Bet v 1-, Der p 1–, and Pyr c 5–specific, cytokine-secreting $CD4^+$ T cells from 15 healthy individuals out of pollen season. (B) Frequency of Der p 1–specific T cells in six allergic and six healthy, and Bet v 1–specific T cells in three allergic and three healthy individuals by ELISPOT assay. *, $P < 0.01$; **, $P < 0.001$.

secreting T cells. There was no difference between different allergens. T cells purified by certain antigen stimulation did not show any cross-reactivity against control antigens (Fig. 2 B). All three subsets purified by Bet v 1 stimulation responded to Bet v 1, but not to tetanus toxoid and Der p 1. Similarly, Der p 1–specific T cell subsets showed proliferative response to Der p 1, but not to tetanus toxoid and Bet v 1 as control antigens. Although they did not proliferate by antigen stimulation, IL-10–secreting T cells used IL-2, IL-4, IL-7, and IL-15 as growth factors and showed significant proliferation (Fig. 2 C). Together with the quantitative cytokine mRNA profiles of freshly purified cells, these data demonstrate that allergen-specific Tr1-, Th1-, and Th2-like cells can be purified from human peripheral blood.

Balance between Allergen-specific Tr1 Cells and Th2 Cells Characterizes Healthy and Allergic Immune Response. As it was possible to purify single allergen-specific Th1-, Th2-, and Tr1-like cells, their frequency and functional properties were investigated in the next step. The frequency of T cell subsets specific to different mucosal allergens was compared in healthy and allergic individuals using two different techniques. Recombinant major allergens of house dust mite (Der p 1) and birch pollen (Bet v 1) were used as aeroallergens, and pear (Pyr c 5) and hazelnut (Cor a 1) were used as food antigens to analyze the frequency of specific Th1-, Th2-, and Tr1-like cells. Although specific T cells that belong to all three subsets were detectable in both healthy and allergic individuals, allergen-specific IL-10–secreting T cells were the predominant subset in healthy individuals. We found similar results for each allergen. In contrast, an increased frequency of IL-4–secreting T cells was observed in allergic patients (Fig. 3 A). ELISPOT was used as an alternative method in allergen-stimulated PBMC cultures and demonstrated a similar frequency distribution of Der p 1– and Bet v 1–specific T cell subsets (Fig. 3 B).

As aforementioned, all three subsets of single allergen-specific T cells are present in both healthy and allergic individuals. Accordingly, their role on allergen-induced T cell proliferation and whether this is influenced by changing their ratios was investigated. We assayed the allergen-induced proliferation of IL-4– and IL-10–secreting T cells by adding those purified cells back into autologous PBMCs. First, the antigen-specific suppressor effect of IL-10–secreting T cells was analyzed (Fig. 4 A). Bet v 1– and Der p 1–specific IL-4– and IL-10–secreting T cells were separately purified from the same healthy individuals. Their frequency was increased up to 10 times higher than initial levels in PBMCs. PBMCs alone did not show allergen-induced T cell proliferation, which was achieved by increasing the numbers of allergen-specific IL-4–secreting T cells. Der p 1–specific IL-10–

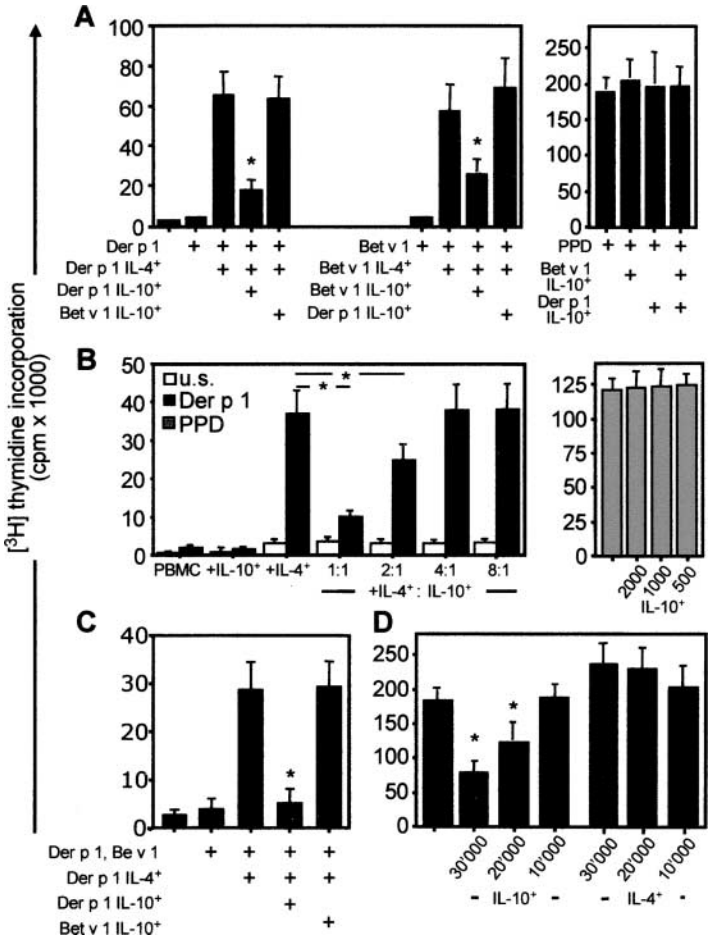

Figure 4. Antigen-specific suppression by Tr1 cells. (A) Der p 1–specific and Bet v 1–specific IL-4–secreting and IL-10–secreting T cells were purified from healthy individuals. Their frequency was calculated in $CD4^+$ T cells, and 2×10^5 PBMCs were immediately reconstituted by increasing their frequency by 10 times (IL-4–secreting T cells, 0.02–0.2%; IL-10–secreting T cells, 0.05–0.5%). Cells were stimulated with the respective antigens and PPD. (B) Der p 1–specific IL-4–secreting and IL-10–secreting T cells were purified and in vitro expanded by IL-2/IL-4 and IL-2/IL-15, respectively. 2×10^5 PBMCs were enriched with 1,000 Der p 1–specific IL-4– or IL-10–secreting T cells or their combinations in IL-4–secreting/IL-10–secreting T cell ratios 1,000/1,000 (1:1), 1,000/500 (2:1), 1,000:250 (4:1), and 1,000:125 (8:1). Cells were stimulated with 0.3 μM Der p 1 or 1 μg/ml PPD. Der p 1–specific, IL-10–secreting T cells added to PPD-stimulated PBMC cultures at indicated numbers did not show any suppression. (C) The same experimental design as in A was used, and cells were stimulated with both Der p 1 and Bet v 1 (0.3 μM each). (A–C) [3H]Thymidine incorporation was determined after 5 d. The same results were obtained in three other experiments. *, $P < 0.001$. (D) 10^5 PBMCs were stimulated with anti-CD3 in the presence of different amounts of IL-10–secreting and IL-4-secreting T cells. [3H]Thymidine incorporation was determined after 3 d. Data represent two different experiments. *, $P < 0.01$.

secreting T cells only suppressed Der p 1–stimulated, but not Bet v 1– or PPD-stimulated proliferation. Similarly, Bet v 1–specific IL-10–secreting T cells only suppressed Bet v 1–stimulated, but not Der p 1– or PPD-stimulated proliferation. There was no cross-suppression of Der p 1–specific IL-10–secreting cells on Bet v 1 stimulation and Bet v 1–specific IL-10–secreting T cells on Der p 1 stimulation, as well as both IL-10–secreting T cells on PPD stimulation.

The retention of suppressive activity of Tr1 cells after expansion ex vivo is obviously an absolute prerequisite for usage in a possible cellular therapeutic approach. We tested whether in vitro–expanded IL-10–secreting T cells maintain their suppressive activity. PBMCs of healthy individuals showed no or very limited proliferative response to Der p 1. The addition of in vitro–expanded Der p 1–specific IL-4, but not IL-10–secreting T cells significantly enhanced Der p 1–induced proliferation (Fig. 4 B). Increased numbers of in vitro–expanded IL-10–secreting T cells significantly suppressed IL-4–secreting T cell–mediated proliferation in Der p 1–stimulated PBMCs. A 1:1 ratio of effector (IL-4–secreting) to suppressor (IL-10–secreting) T cells was required for full suppression of T cell proliferation. In contrast, PPD-stimulated proliferation was not suppressed by Der p 1–specific IL-10–secreting T cells.

To test whether the IL-10–secreting T cells induce bystander suppression on other T cells in their vicinity, we cultured Der p 1–specific, IL-4–secreting cells with freshly purified Der p 1– or Bet v 1–specific IL-10–secreting cells in the presence of both Der p 1 and Bet v 1 (Fig. 4 C). Der p 1–specific, IL-4–secreting T cell proliferation was suppressed by Der p 1–, but not Bet v 1–specific Tr1 cells. As few as 1,000 Tr1 cells were able to induce antigen-specific suppression in 200,000 PBMCs. High numbers of freshly purified Tr1 cells (at least 20,000 in 100,000 PBMCs) resulted in a nonspecific suppressive activity on anti-CD3–stimulated T cells (Fig. 4 D). In comparison, IL-4–secreting T cells did not exert any suppressive effect on anti-CD3 stimulation. These data demonstrate allergen-specific suppressor activity of Tr1 cells and the importance of the balance between allergen-specific Th2 and Tr1 cells for allergen-induced T cell activation.

Allergen-specific Tr1 Cells Use Multiple Suppressor Factors. Molecules that may play a role on suppressive mechanisms of allergen-specific IL-10–secreting T cells were analyzed

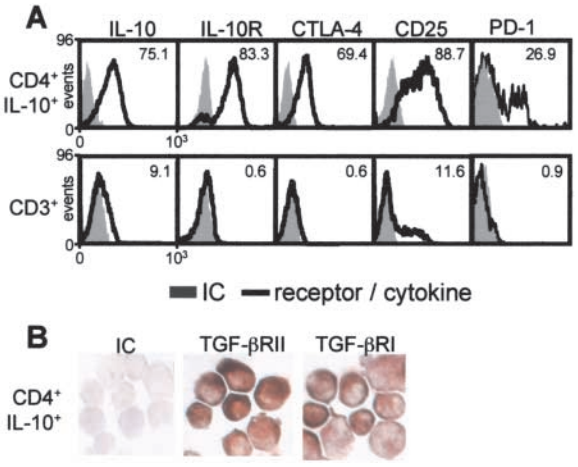

Figure 5. Expression of suppressor molecules on IL-10–secreting T cells. (A) Immediately after purification, antigen-specific IL-10–secreting T cells of healthy individuals were analyzed for intracytoplasmic IL-10, surface IL-10R, CTLA-4, CD25, and PD-1 by flow cytometry. Data are compared with $CD3^+$ T cells of the same donor stimulated with the same antigen. (B) TGF-β RI and RII expression by immunohistology. (A and B) Same results were obtained in three independent experiments.

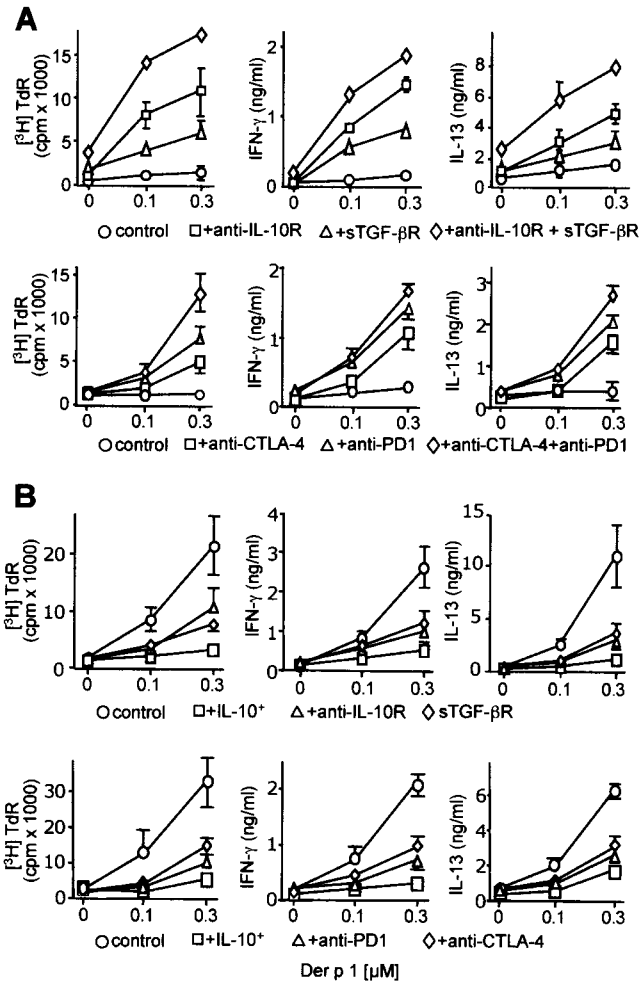

Figure 6. Multiple suppressive mechanisms play a role in peripheral allergen tolerance. (A) Endogenous IL-10, TGF-β, or both as well as CTLA-4, PD-1, or both were neutralized in Der p 1–stimulated PBMCs of healthy individuals. [3H]Thymidine incorporation (TdR), IFN-γ, and IL-13 were determined at day 5. (B) Der p 1–specific proliferation of PBMCs from house dust mite allergic patients was suppressed by 10 times increased frequency of Der p 1–specific, IL-10–secreting T cells ($IL\text{-}10^+$). The activity of IL-10, TGF-β, CTLA-4, and PD-1 were neutralized. [3H]Thymidine incorporation (TdR), IFN-γ, and IL-13 were determined at day 5. (control) Isotype control antibody. (A and B) Same results were obtained in four independent experiments (two Bet v1 and two Der p 1) all performed with freshly purified cells without in vitro expansion. Bet v 1 stimulation in birch pollen–allergic individuals gave similar results. All of the neutralization experiments were significantly different from the control and IL-10–secreting T cell–suppressed condition at 0.3 μM allergen doses. $P < 0.001$.

on freshly purified cells (Fig. 5). IL-10–secreting T cells expressed high amounts of IL-10, IL-10Rα chain, TGF-β receptors I and II, CTLA-4, CD25, and PD-1, suggesting that multiple suppressor factors may play a role on suppression of allergen-specific Th2 cells by Tr1 cells in healthy individuals.

Neutralization experiments revealed that all four suppressive mechanisms may play a role in suppression of allergen-specific Th2 cells. Der p 1– or Bet v 1–stimulated PBMCs of healthy individuals did not show any T cell proliferation. In both cases, neutralization of IL-10 and TGF-β activity significantly enhanced antigen-induced proliferation as well as IL-13 and IFN-γ production (Fig. 6 A). In addition, two other mechanisms apparently function in healthy immune response to allergens because neutralization of CTLA-4 or PD-1 significantly enhanced T cell proliferation and IFN-γ and IL-13 secretion in healthy individuals. In allergic individuals, Der p 1–induced T cell proliferation was significantly high in PBMCs of house dust mite allergic donors. A very clear suppression was achieved by increasing the frequency of Der p 1–specific, IL-10–secreting T cells. This suppression was partially inhibited by blocking of IL-10R, TGF-β, CTLA-4, or PD-1 (Fig. 6 B). In these experiments, IL-10R–blocking mAbs may block IL-10R on the APCs as well as on Tr1 cells; in addition, TGF-β secreted from both Tr1 cells and APCs can be neutralized by sTGF-βR. CTLA-4 and PD-1 are blocked particularly on the surface of Tr1 cells. These data demonstrate that IL-10, TGF-β, CTLA-4, and PD-1 cooperate in the suppression of immune response to allergens.

Discussion

The present work demonstrates that immune response to allergens in health and disease is the result of a balance between allergen-specific Tr1 cells and allergen-specific Th2 cells. Active regulation has emerged as a very essential mechanism for both inducing and equally importantly maintaining specific immunological nonresponsiveness. By analyzing Tr1 cells specific to various food or inhalant antigens, we demonstrated that similar mechanisms take place in healthy immune response to mucosal allergens. The IL-10–secreting allergen-specific T cells represented the predominant subset with significantly high frequency in comparison to IL-4– and IFN-γ–secreting T cells in healthy individuals. Both ELISPOT for cytokines and purification of cytokine-secreting T cells gave similar frequency numbers. Mechanisms that control the in vivo shift between allergen-specific T cell subsets remain to be elucidated. It can be hypothesized that a switch in cytokine profile and apoptosis of effector T cells may play a role in the generation of an atopic phenotype (29, 30). The stability of cytokine profile in differentiated effector and memory T cell subsets in humans is not fully known, and recent studies have demonstrated that lineage-committed memory T cell subsets are responsive to cytokine signals of the opposing lineage (30, 31). In addition, Tr1 cells do not appear to be anergic and may efficiently expand in vivo (32) as they are shown to proliferate by IL-2, IL-4, IL-7, and IL-15 in the present work.

There is clear evidence from various animal models and human studies for an active mechanism of immune suppression, whereby a distinct subset of T cells inhibits the activation of conventional T cells in the periphery (7, 13, 14, 33). This Tr cell population has been determined as $CD4^+CD25^+$ T cells. They can prevent the development of autoimmunity, indicating that the normal immune system contains a population of professional regulatory T cells. Elimination of $CD4^+CD25^+$ T cells leads to spontaneous development of various autoimmune diseases, such as gastritis or thyroiditis, in genetically susceptible hosts. The frequency of $CD4^+CD25^+$ Tr cells is ~10–15% of $CD4^+$ T cells, whereas the frequency of IL-10–secreting T cells of a single allergen specificity ranges between 0.1 and 0.007% of $CD4^+$ T cells. This shows that the frequency of single allergen-specific Tr1 cells, which are also $CD4^+$ $CD25^+$, ranges between 1 in 1,000 and 1 in 20,000 of the whole $CD4^+$ $CD25^+$ Tr cell population.

Although many aspects of the mechanisms by which suppressor cells exert their effects remain to be elucidated, it is well established that Tr cells suppress immune responses via cell-to-cell interactions and/or the production of IL-10 and TGF-β (10, 11, 34, 35). Tr1 cells specific for a variety of antigens arise in vivo, but may also differentiate from naive $CD4^+$ T cells in the presence of IL-10 in vitro (36). The nonspecific T cell suppressor activity of IL-10 and TGF-β has been consistently reported in experiments with high amounts of exogenously added suppressor cytokines (21, 25). However, the present work demonstrates that Tr1 cells display antigen-specific suppressor activity in very low numbers. If the number of cells exceed a threshold that provides sufficient quantities of suppressor signals, apparently they show nonspecific suppression. Depending on their frequency, the first T cell that contacts the APC may be very critical in the subsequent decision to stimulate or suppress the specific immune response. If the first T cell to contact the APC is a Tr1 cell, it may silence or regulate the maturation of APC. IL-10 down-regulates the antigen-presenting capacity, such as HLA-DR expression, costimulatory molecules, and several cytokines in dendritic cells and monocytes/macrophages (37). Recently, differentiation of a distinct dendritic cell subset in the presence of IL-10 has been demonstrated that induces tolerance through the generation of Tr1 cells (38). In addition, exposure of mature pulmonary dendritic cells to respiratory allergens stimulated the development of Tr1-like cells, which was dependent on dendritic cell IL-10 production (39). Together, these findings suggest that IL-10–secreting T cells may regulate the functional state of APCs in a way that these APCs can now promote the generation of Tr1 cells.

$CD4^+CD25^+$ T cells are the only lymphocyte subpopulation in mice and humans that express CTLA-4 constitutively. The expression apparently correlates with the suppressor function of CTLA-4. As demonstrated in the present work, the blocking of CTLA-4 activity of Tr1 cells

reverses suppression in cocultures of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells (40). Similarly, the treatment of mice that were recipients of $CD4^+CD45RB^{low}$ T cells with CTLA-4–blocking agents, abrogated the suppression of inflammatory bowel disease (41). These studies indicate that signals that result from the engagement of CTLA-4 by its ligands, CD80 or CD86, are required for the induction of suppressor activity. Under some circumstances, the engagement of CTLA-4 on the $CD4^+CD25^+$ T cells by antibody or by CD80/CD86 might lead to inhibition of the TCR-derived signals that are required for the induction of suppressor activity.

A subset of human Tr1 cells expressed functional PD-1 in the present work. PD-1 is an immunoreceptor tyrosine-based inhibitory motif–containing receptor expressed upon T cell activation. PD-1–deleted mice develop autoimmune diseases, suggesting an inhibitory role for PD-1 in immune responses (42). Members of the B7 family, PD-ligand (L)1 and PD-L2, are ligands for PD-1. PD-1/PD-L engagement on murine CD4 and CD8 T cells results in inhibition of proliferation and cytokine production. T cells stimulated with anti-CD3/PD-L1Fc-coated beads display dramatically decreased proliferation and IL-2 production (43).

Collectively, our results indicate that the control of Th2 immune response against naturally exposed harmless environmental antigens is mediated by Tr1 cells in humans. Effector (allergen-specific Th2) and suppressor (allergen-specific Tr1) T cells exist in both healthy and allergic individuals in certain amounts. Their ratio determines the development of a healthy or an allergic immune response. These data may explain the spontaneous development and spontaneous healing of allergic diseases. Although in low frequency, the existence of potential suppressive allergen-specific Tr1 cells in allergic individuals suggests a possible way of treatment. The knowledge of this cellular and molecular basis is pivotal in understanding the mechanisms of immune tolerance or allergy development against harmless environmental proteins.

We thank Drs. C.H. Heusser for anti–IL-4 and anti–IFN-γ mAbs, K. Moore for anti–IL-10R–blocking mAb, and S. Vieths and D. Lüttkopf for rCor a 1 and rPyr c 5. We thank Drs. F. Melchers and O. Alpan for critical reading the manuscript.

This work was supported by the Swiss National Foundation grants 32-100266, 31-65436, and 32-65661.

Submitted: 1 December 2003
Accepted: 7 April 2004

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6.3 A second step of chemotaxis after transendothelial migration: keratinocytes undergoing apoptosis release IFN-γ-inducible protein 10, monokine induced by IFN-γ, and IFN-γ-inducible α-chemoattractant for T cell chemotaxis toward epidermis in atopic dermatitis.

J Immunol., 2003. 171: 1078-1084

A Second Step of Chemotaxis After Transendothelial Migration: Keratinocytes Undergoing Apoptosis Release IFN-γ-Inducible Protein 10, Monokine Induced by IFN-γ, and IFN-γ-Inducible α-Chemoattractant for T Cell Chemotaxis Toward Epidermis in Atopic Dermatitis[1]

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Activation and skin-selective homing of T cells and their effector functions in the skin represent sequential immunological events in the pathogenesis of atopic dermatitis (AD). Apoptosis of keratinocytes, induced mainly by T cells and mediated by IFN-γ and Fas, is the essential pathogenetic event in eczema formation. Keratinocyte apoptosis appears as activation-induced cell death in AD. By IFN-γ stimulation, chemokines such as IFN-γ-inducible protein 10, monokine induced by IFN-γ, and IFN-γ-inducible α-chemoattractant are strongly up-regulated in keratinocytes. These chemokines attract T cells bearing the specific receptor CXCR3, which is highly expressed on T cells isolated from skin biopsies of AD patients. Accordingly, an increased T cell chemotaxis was observed toward IFN-γ-treated keratinocytes. Supporting these findings, enhanced IFN-γ-inducible protein 10, monokine induced by IFN-γ, and IFN-γ-inducible α-chemoattractant expression was observed in lesional AD skin by immunohistochemical staining. These results indicate a second step of chemotaxis inside the skin after transendothelial migration of the inflammatory cells. Keratinocytes undergoing apoptosis in acute eczematous lesions release chemokines that attract more T cells toward the epidermis, which may further augment the inflammation and keratinocyte apoptosis. *The Journal of Immunology,* **2003, 171: 1078–1084.**

Chemokines are a superfamily of small structurally related proteins that regulate the traffic of lymphocytes, dendritic cells, monocytes, neutrophils, and eosinophils. They are classified into C, CC, CXC, and CX3C subfamilies, based on the position of characteristic structure determining cysteine residues within the N-terminal part of the proteins (1–3). Another classification distinguishes chemokines in inflammatory (inducible) and homeostatic (constitutive, housekeeping or lymphoid) chemokines (4–9). Homeostatic chemokines are constitutively produced in discrete microenvironments within lymphoid or nonlymphoid tissues including skin and mucosa. They are responsible for maintaining physiological traffic and positioning of cells that mainly belong to the adaptive immune system. Inflammatory chemokines are expressed after stimulation by proinflammatory cytokines or after contact with pathogenic agents. This causes the recruitment of effector cells, such as monocytes, granulocytes, and effector T cells (4). IFN-γ-inducible protein 10 (IP-10,[3] CXCL10), monokine induced by IFN-γ (Mig, CXCL9), and IFN-γ-inducible α-chemoattractant (iTac, CXCL11) belong to the group of inflammatory chemokines. They are all induced by IFN-γ, which inhibits the expression of most other chemokines (10, 11), and these three chemokines share a common receptor, CXCR3 (12).

Atopic dermatitis (AD) is a chronic inflammatory skin disease that frequently predates the development of allergic rhinitis or asthma (13, 14). Elevated IgE levels and eosinophilia in AD suggest increased expression of Th2-type cytokines. The majority of allergen-specific T cells derived from skin lesions that have been provoked in AD patients by epicutaneous allergen application or peripheral blood skin- homing T cells produce predominantly Th2 cytokines such as IL-4, IL-5, and IL-13 (15–18). Previously, such a polarized Th2 cytokine pattern was regarded as a specific feature reflecting immune dysregulation in AD. However, recent studies have demonstrated that IFN-γ predominates over IL-4 in chronic skin lesions and older patch test reactions in AD, whereas IL-5 and IL-13 still remain at high levels (19–22).

Lesional AD skin is characterized with dermal mononuclear cell infiltrate and spongiosis in the epidermis. Apoptosis of keratinocytes induced by T cells and mediated by IFN-γ and Fas (CD95) is a crucial event in the transition from activation of the immune system to the manifestation of eczematous dermatitis (23). Apparently, keratinocyte apoptosis is an activation-induced cell death, because IFN-γ up-regulates Fas, ICAM-1, and HLA-DR and renders keratinocytes susceptible to apoptosis (24). Induction of keratinocyte apoptosis by skin-infiltrating T cells, subsequent cleavage of E-cadherin, and resisting desmosomal cadherins represent molecular events in spongiosis formation (25).

We investigated whether T cell-keratinocyte interaction contributes to the intensity of skin inflammation and tissue injury mechanisms. Here, we demonstrate that keratinocytes undergoing T cell- and particularly IFN-γ-mediated activation-induced cell

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Received for publication January 8, 2003. Accepted for publication May 13, 2003.

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[1] This study was supported by Swiss National Foundation Grant 32.65661.01.

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[3] Abbreviations used in this paper: IP-10, IFN-γ-inducible protein 10 (CXCL10); Mig, monokine induced by IFN-γ (CXCL9); iTac, IFN-γ-inducible α-chemoattractant (CXCL11); AD, atopic dermatitis; FL, fluorescein labeled; LC, Red640LC labeled; MCP, monocyte chemoattractant protein; sFas, soluble Fas.

0022-1767/03/$02.00

death release chemokines such as IP-10, Mig, and iTac. These chemokines play a role in a second step of chemotaxis from the dermis to the epidermis that increases the severity of tissue injury and keratinocyte apoptosis in the AD skin.

Materials and Methods

Subjects

Nineteen patients with AD (19–68 years of age) were diagnosed according to standard criteria of Hanifin and Rajka (26). They were all allergic to at least three environmental allergens, and serum IgE was >500 U/ml. Ten healthy subjects (22–42 years of age) had no allergic disease and had normal levels of serum IgE. The study was approved by the Ethical Committee of Davos.

Abs and reagents

All fluorescent- or biotin-labeled mAbs for flow cytometry analysis were purchased from Immunotech (Marseilles, France) or BD PharMingen (San Diego, CA). Anti-CD45RO, anti-CD45RA magnetic microbeads for MACS were from Miltenyi Biotec (Bergisch Gladbach, Germany). Human IFN-γ, IL-2, and IL-4 were provided by Novartis (Basel, Switzerland). Ethidium bromide was from Sigma-Aldrich (St. Louis, MO). Soluble Fas ligand and TNF-α were from Alexis Biochemicals (San Diego, CA). Abs to human IP-10, Mig, and iTac for immunohistochemistry were purchased from PeproTech EC (London, U.K.).

Keratinocyte culture

Primary human keratinocytes (pooled normal human epidermal keratinocytes from neonatal skin) were purchased from BioWhittaker (Walkersville, MD) and grown in keratinocyte growth medium (KGM-2) from the same company supplemented with bovine insulin, hydrocortisone, human recombinant epidermal growth factor, 30 μg/ml bovine pituitary extract, 100 μg/ml gentamicin, 1000 ng/ml amphotericin B, epinephrine, transferrin, and calcium. Hydrocortisone was not added in experiments.

Human HaCaT keratinocytes, a gift from N. E. Fusenig (German Cancer Research Center, Heidelberg, Germany), (27), were grown in RPMI 1640 supplemented with 1 mM L-glutamine, sodium pyruvate, nonessential amino acids, and 10% FCS (all from Life Technologies, Basel, Switzerland).

Real time RT-PCR mRNA expression analysis

Total RNA from keratinocytes was extracted using the RNeasy kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. During this procedure, a DNase digestion with the RNase-free DNase set for use with RNeasy/QIAamp columns (Qiagen) was used. RNA was reverse transcribed using Omniscript reverse transcriptase (Qiagen). The resulting cDNA was analyzed by real time PCR (Light Cycler; Roche Diagnostics, Mannheim, Germany). The probes for IP-10 were: Red640LC-labeled (LC), 5′-LC Red640-act gga ggt tcc tct gct gta ggc tc p; and fluorescein-labeled (FL), 5′-aat cgc agt ttg att cat ggt gct ga x. The primers for IP-10 were: forward, 5′-gac att cct caa ttg ctt aga cat a; and reverse, 5′-aat gat ctc aac acg tgg aca a. The probes for Mig were: LC, 5′-LC Red640-cct aca caa ttc act gaa cct ccc ctg p; and FL, 5′-ggt tta aat tct ggc cac aga caa cct c x. The primers for Mig were: reverse, 5′-tag ata aga cgt tcg ggt ggg at and forward, 5′-cgg tta gtg gaa gca tga ttg g. The probes for iTac were: LC, 5′-LC Red640-gac agc gtc ctc ttt tga aca tgg g p; and FL, 5′-ctg ctt tta ccc cag ggc cta tgc x. The primers for iTac were: forward, 5′-tgg ctg tga tat tgt gtg cta ca; and reverse, 5′-ctt cga ttt ggg att tag gca. All primers and probes were designed and obtained from TIB MOLBIOL (Berlin, Germany). For amplification of the PCR product, the Light Cycler-Fast Start DNA Master Hybridization Probes (Roche Diagnostics) were used according to the manufacturer's instructions. The forward primer sequence for GAPDH was 5′-gct gat gat ctt gag gct gtt g-3′, and the reverse primer sequence was 5′-ctt cgc tct ctg ctc ctc ct-3′. The GAPDH was amplified in the real time PCR using the Light Cycler-Fast Start DNA Master SYBR Green I (Roche Diagnostics).

Quantification of the amplicons in each well was determined according to the comparative threshold cycle number (*Ct*) method as previously described (Applied Biosystems, Foster City, CA). Briefly, the formula for each sample is $2E - (Ct_{\text{target}} - Ct_{\text{standard}})$. This yields a quantification of the target PCR products relative to the PCR products for the internal calibration (GAPDH) and unstimulated control keratinocytes. The results were plotted on a log scale.

RNase protection assay mRNA expression analysis

Total RNA was extracted using a guanidine isothiocyanate-acid phenol protocol. For simultaneous RNase protection assay of RANTES (CCL5), IP-10, monocyte chemoattractant protein (MCP-1, CCL2), and IL-8, the multiprobe template set hCK5 was used; hCR-6 was used for the detection of CXCR1, CXCR2, CXCR3, and CXCR4 (BD PharMingen) according to the manufacturer's instructions. The unprotected RNA was digested using a RNase T1/A mix. The digested RNA was purified and loaded on a 6% urea gel. The dried radioactive gels were exposed to imager plates and visualized by an imager system (FLA-3000; Raytest, Urdorf, Switzerland). RNase protection assay bands were quantified by using the AIDA software (Raytest).

T cell cultures

PBMC were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood of normal donor and cord blood (28). Cells were washed three times and resuspended in DMEM (Life Technologies, Switzerland), supplemented as described (16). $CD45RO^+$ and $CD45RA^+$ cells were negatively selected with the MACS magnet activated cell separation system (Miltenyi Biotech) (16, 17). In brief, anti-CD14 and anti-CD19 depleted cells were incubated with MACS microbead-conjugated anti-CD45RO and anti-CD16 mAbs and anti-CD8. For the differentiation of Th1 and Th2 cells, freshly isolated cord blood $CD4^+CD45RA^+$ T cells were suspended in RPMI 1640 medium supplemented as described (16). The cells were cultured in 48-well plates at a cell density of 10^5/ml. They were stimulated with the combination of mAbs to T cell surface molecules anti-CD2 (two mAbs, 4B2 and 6G4 each 0.5 μg/ml), anti-CD3 (1 μg/ml), and anti-CD28 (1 μg/ml), and IL-2 (20 ng/ml). For Th1 differentiation, human IL-12 (10 ng/ml) and neutralizing anti-IL-4 mAb (10 μg/ml) were added to individual wells (29). For Th2 differentiation, human IL-4 (25 ng/ml) and neutralizing anti-IL-12 (10 μg/ml) were used (29). The growing cell cultures were expanded with fresh culture medium containing human IL-2. After 12 days, the cells were harvested, washed, and then restimulated with mAbs to CD2/CD3/CD28, and cytokine patterns of differentiated cells were determined by flow cytometry and ELISA.

To isolate T cells from epidermis of lesional biopsies of AD patients, epidermis pieces (0.5–1 mm) of the skin biopsy specimens from 3- to 4-day-old lesions of four chronic AD patients were minced with two scalpels (no proteolytic enzymes were used). Pieces of epidermis were stimulated in complete RPMI 1640 with 25 U/ml IL-2 and anti-CD2, anti-CD3, and anti-CD28 mAb. Growing T cells were expanded in medium containing IL-2 (purity was 100% as assessed by flow cytometry). After 7–10 days, the cytokine profile and chemokine receptors were characterized (19). The supernatants were collected 48 h after anti-CD2, anti-CD3, and anti-CD28 mAb stimulation; frozen; and used for the chemokine expression experiments or ELISA measurements of cytokines.

Quantification of cytokines by ELISA

The cytokine profiles of the T cells that were isolated from skin biopsies were evaluated in a sandwich ELISA (16, 28) by measuring IL-4, IL-5, IL-13, and IFN-γ. The sensitivity of IFN-γ ELISA was $\leq$10 pg/ml (mAb and IFN-γ standard were gifts from Dr. S. S. Alkan, Novartis). The sensitivity of IL-4 ELISA was $\leq$20 pg/ml (mAb and IL-4 standard were provided by Dr. C. H. Heusser, Novartis). The detection limit of the IL-5 ELISA was 50 pg/ml (mAb and IL-5 standard were from BD PharMingen). The sensitivity of the IL-13 ELISA was 100 pg/ml (mAb and IL-13 standard were from BD PharMingen).

Chemotaxis assay

$CD45RO^+$ T cells (1×10^6)/ml were stimulated with a combination of soluble anti-CD2, anti-CD3, and anti-CD28 mAb. The cells were stained with Cell Tracker Green CMFDA (Molecular Probes, Eugene, OR; 5 μM in medium) for 45 min under growth conditions.

Keratinocytes were grown in a 24-well plate until a confluence of ~80% was reached. A fluoro-block insert (BD Biosciences, Basel, Switzerland) with a pore size of 3 μm was used to separate the fluorescence-labeled T cells from the keratinocytes at the bottom of the well. Keratinocytes were pretreated for 12 h with 10 ng/ml IFN-γ or 10 ng/ml TNF-α. Migration occurred through the pores of the membrane and was measured by the amount of fluorescence in the lower part of the well with an imager system, FLA-3000 (Raytest).

Immunohistochemistry

Biopsy specimens were taken from 3- to 4-day-old lesions of chronic AD patients or from normal skin of healthy individuals. Frozen 4-μm skin

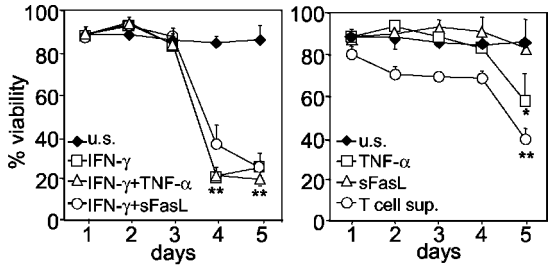

FIGURE 1. Role of T cells and cytokines on keratinocyte death. Primary human keratinocytes were incubated with 10 ng/ml IFN-γ, 10 ng/ml TNF-α, 10 ng/ml sFas ligand (sFasL), and 50% (v/v) T cell supernatant (sup., 48-h anti-CD2/CD3/CD28 mAb-activated AD skin T cell supernatant) and left unstimulated (u.s.). KC viability was monitored by ethidium bromide exclusion and flow cytometry. One representative of five experiments with primary human keratinocytes is shown. Same results were obtained with HaCaT keratinocytes. Standard deviation of triplicate cultures is shown. **, $p < 0.001$; *, $p < 0.05$.

sections were fixed with 4% paraformaldehyde for 20 min. Endogenous peroxidases were quenched with a 0.3% H_2O_2 solution in methanol. Nonspecific binding was blocked with 10% normal goat serum (Chemicon, Temecula, CA) and then incubated for 2 h with the appropriate dilution of Abs, anti-human CD4 (BD PharMingen), anti-human IP-10, anti-human iTac, or anti-human Mig (all from PeproTech EC) or rabbit IgG as isotype control. Sections were then treated with biotin-labeled goat anti-rabbit IgG and stained with avidin-biotin-peroxidase (Vector, Burlingame, CA), followed by 3,3′-diaminobenzidine (Sigma-Aldrich) development.

Flow cytometry

Keratinocytes or T cells were incubated with appropriate FITC- or PE-labeled mAb for 30 min at 4°C and then fixed in 2% paraformaldehyde (pH 7.4). Negative control cells were prepared in a similar fashion with isotype control IgG1-FITC/PE mAb. Fluorescence intensity was measured by flow cytometry (EPICSTMXL-MCL; Beckmann Coulter, Nyon, Switzerland). KC viability was monitored by ethidium bromide (1 μM; Sigma-Aldrich) uptake and flow cytometry as previously described (30).

Statistical analysis

Student's *t* test was used for statistical analysis. Data are presented as the mean ± SD of triplicate cultures.

Results

IFN-γ-, TNF-α-, and T cell-mediated death of human keratinocytes

Primary human keratinocytes or HaCaT keratinocytes were cultured with IFN-γ, TNF-α, soluble Fas (sFas) ligand, and anti-CD2/CD3/CD28 mAb stimulated supernatant of T cells isolated from AD skin, and cell viability was measured (Fig. 1). A significantly increased keratinocyte death was induced after 5 days by supernatants of stimulated T cells isolated from AD skin and conditions, which contain IFN-γ ($p < 0.001$). TNF-α induced a relatively low level and late keratinocyte death. Triggering of Fas alone on keratinocytes did not induce keratinocyte death. In our previous studies, IFN-γ and sFas-ligand were identified as keratinocyte apoptosis-inducing factors within the activated T cell supernatants. The type of T cell-induced keratinocyte death was also shown to be apoptosis by using several different apoptosis-specific methods (23, 25).

IFN-γ induces IP-10, Mig, and iTac in human keratinocytes

Because T cells are essential players in keratinocyte apoptosis, factors that attract T cells toward and into the epidermis of lesional AD skin are further investigated. Because IFN-γ and TNF-α are two major proinflammatory cytokines in eczematous skin, we investigated whether they affect release of chemokines by keratinocytes. In

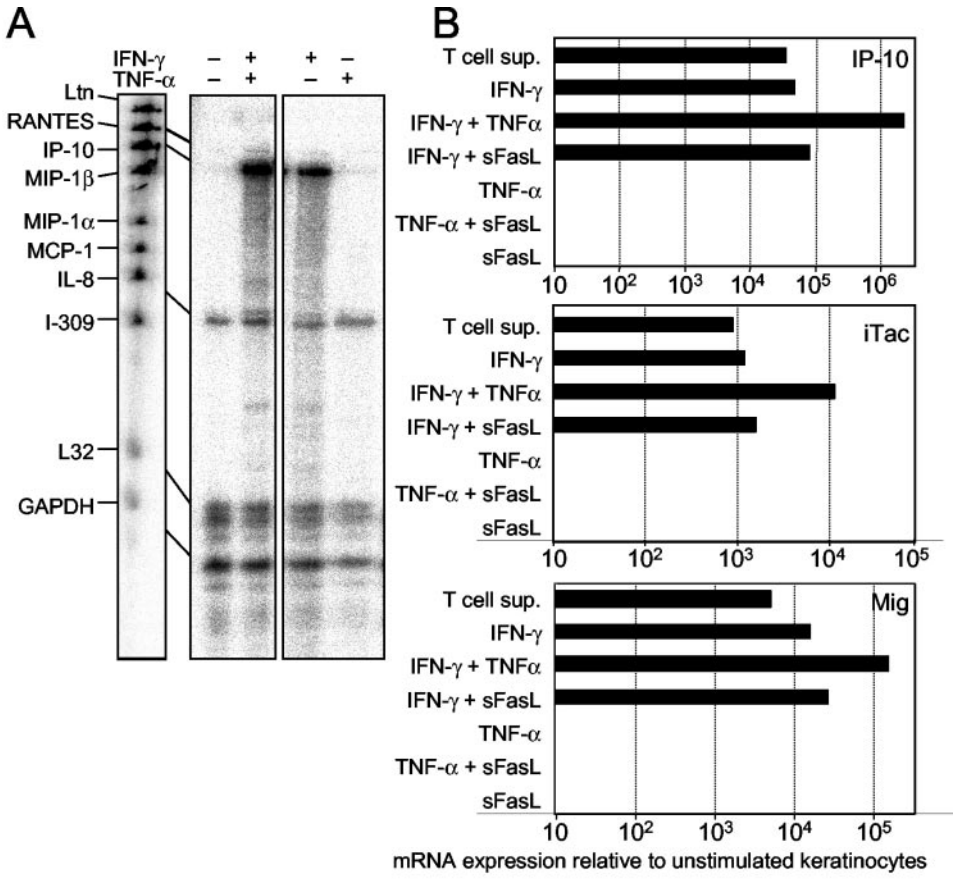

FIGURE 2. IFN-γ induces IP-10, Mig, and iTac in human keratinocytes. *A*, RNase protection assay of cultured primary keratinocytes shows constitutive expression of IL-8 mRNA and induction of IP-10 mRNA after treatment with 10 ng/ml IFN-γ or IFN-γ and an additional 10 ng/ml TNF-α. *B*, Real time RT-PCR shows up-regulation of IP-10, iTac, and Mig mRNA after treatment of primary keratinocytes with 50% (v/v) T cell supernatant (sup.; 48 h anti-CD2/CD3/CD28 mAb stimulated AD skin T cell supernatant). IFN-γ, TNF-α, and sFas ligand (sFasL) were used at 10 ng/ml. The target PCR products are quantified relative to the PCR products for the internal calibration (GAPDH) and unstimulated control keratinocytes. One representative is shown. Same results were obtained in two experiments from two different donors. MIP, macrophage-inhibitory protein; Ltn., lymphotactin; L32, housekeeping gene that encodes L32 ribosomal protein.

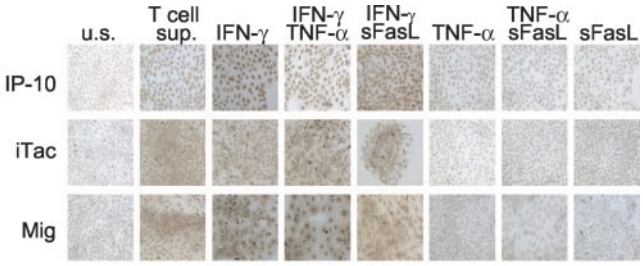

FIGURE 3. IP-10, Mig, and iTac are expressed by IFN-γ stimulation in keratinocytes. Immunohistochemical staining of cultured keratinocytes show up-regulation of IP-10, Mig, and iTac after incubation with T cell supernatant and IFN-γ. Primary human keratinocytes were treated with 50% (v/v) T cell supernatant (sup.; 48 h anti-CD2/CD3/CD28 mAb-stimulated AD skin T cell supernatant), IFN-γ, TNF-α, and sFas ligand (sFasL); u.s., unstimulated keratinocytes. IFN-γ, TNF-α, and sFas ligand were all used at 10 ng/ml. All original magnifications are ×200. Same results were obtained in four independent experiments.

RNase protection assays analyzing several chemokines, IL-8 mRNA was constitutively expressed, whereas IP-10 mRNA was up-regulated by IFN-γ in human primary keratinocytes (Fig. 2*A*).

Increased mRNA expression of IP-10, Mig, and iTac was observed by IFN-γ stimulation of primary human keratinocytes. TNF-α had a synergistic effect on the up-regulation of all three chemokines by IFN-γ, without showing any effect alone. Anti-CD2/CD3/CD28 mAb-stimulated AD skin T cell supernatants also up-regulated these three chemokines. Triggering of the Fas by sFasL did not stimulate chemokine expression alone or in addition to IFN-γ and TNF-α (Fig. 2*B*).

To confirm the mRNA expression profiles, we investigated the IP-10, Mig, and iTac protein expression on primary human keratinocytes by immunohistology. An up-regulation of IP-10, Mig, and iTac was observed after stimulation of keratinocytes with supernatant of T cells isolated from AD skin and IFN-γ. TNF-α alone or together with Fas-ligand did not show any effect (Fig. 3).

CXCR3 is expressed on T cells

IP-10, Mig, and iTac share the same receptor, CXCR3. We investigated the expression of CXCR3 on T cells isolated from lesional skin of AD patients, peripheral blood T cells of healthy individuals, and AD patients as well as in vitro differentiated Th1 and Th2 cells. Freshly purified $CD45RO^+$ cells and in vitro differentiated cord blood $CD4^+$ Th1 and Th2 cells expressed CXCR3. Higher expression of CXCR3 was observed on Th1 than on Th2 cells (Fig. 4*A*). We determined CXCR3 expression on peripheral blood T cells of healthy individuals and atopic dermatitis patients by double staining together with an anti-CD3 mAb. CXCR3 was expressed on 63.0 ± 9.3% of T cells from healthy individuals and 70.2 ± 7.2% on T cells of AD patients. There was no significant difference (Fig. 4*B*). Within several chemokine receptors, CXCR3 mRNA was expressed in addition to CXCR4, CXCR5, and CCR7 mRNA on T cells, which were isolated and cultured from skin biopsy samples of AD patients (Fig. 4*C*).

Enhanced T cell chemotaxis toward IFN-γ-treated keratinocytes

The histological hallmark of eczematous lesions in the AD skin is dermal mononuclear cell infiltration and spongioform morphology in the epidermis. As shown in $CD4^+$ T cell staining in Fig. 5*A*, some of the $CD4^+$ T cells have migrated inside the epidermis. Several $CD4^+$ T cells have mainly concentrated in the epidermal area, which shows substantial spongiosis. Because differential chemotactic features of Th1 and Th2 cells have been demonstrated, we analyzed the cytokine profile of T cells, which infiltrate the epidermis of AD lesions. Epidermal T cells were cultured from different AD patients, and their cytokine profile was characterized. Epidermal T cells showed a Th0 cytokine profile with high quantities of IFN-γ, IL-5, and IL-13 and relatively lower amounts of IL-4 (Fig. 5*B*).

To analyze the role of IFN-γ on T cell chemotaxis, we used a Transwell system, in which fluorescence-labeled T cells migrated toward primary human keratinocytes separated with a 3-μm pore size filter. Enhanced migration of T cells toward IFN-γ-treated keratinocytes in comparison with unstimulated or TNF-α treated keratinocytes was observed. There was no difference between $CD4^+$ Th1 and $CD4^+$ Th2 cells. TNF-α-pretreated keratinocytes did not induce either $CD4^+$ Th1 or $CD4^+$ Th2 cell chemotaxis (Fig. 5*C*).

Increased IP-10, Mig, and iTac expression in AD skin

The in vivo relevance of the studied hypothesis was investigated by demonstration of the IFN-γ-induced chemokines in AD skin (Fig. 6). IP-10, Mig, and iTac were stained in lesional AD and normal skin of healthy individuals by immunohistochemistry. Enhanced IP-10 distribution was detected in the suprabasal layer of the epidermis in lesional acute AD skin biopsies. In healthy skin, IP-10 expression was restricted to basal keratinocytes, whereas suprabasal epidermis did not show any expression. In AD skin, Mig and iTac expression was more distributed within the whole epidermis. Similar to IP-10, Mig expression was restricted to basal keratinocytes in healthy skin, whereas stronger expression was observed in suprabasal layers of lesional AD skin. In contrast, iTac

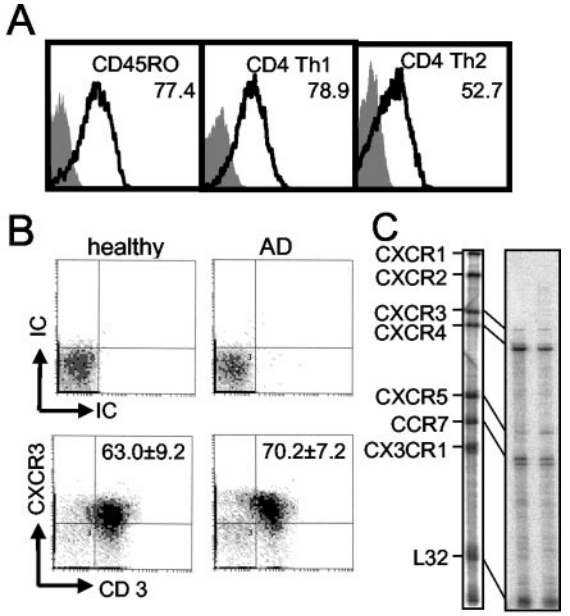

FIGURE 4. CXCR3 is expressed on T cells. *A*, FACS analysis showing that CXCR3 was expressed on CD4 Th1 cells and $CD45RO^+$ cells. CD4 Th2 cells showed a lower expression of CXCR3. One of two experiments with similar results is shown. The percent of $CXCR3^+$ T cells is shown on the right side of each histogram. *B*, FACS analysis of CXCR3 and CD3 on PBMC of seven AD patients and six healthy individuals. One demonstrative FACS datum shown with mean ± SD of all individuals on the upper right quadrant. IC, isotype control Ab. *C*, RNase protection assay showed expression of CXCR3, CXCR4, CXCR5, and CCR7 mRNA in anti-CD2/CD3/CD28 mAb-stimulated skin T cells isolated from lesional epidermis of two different AD patients. L32, housekeeping gene that encodes L32 ribosomal protein.

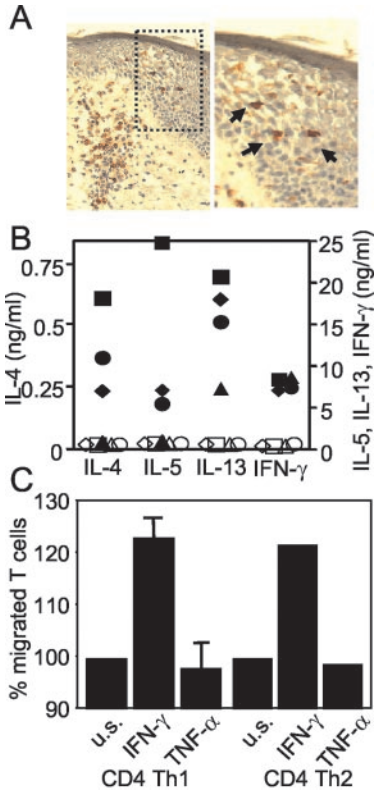

FIGURE 5. Enhanced chemotaxis of $CD4^+$ T cells toward IFN-γ-stimulated keratinocytes. *A*, $CD4^+$ T cells infiltrating the spongiotic epidermis. ×200. Magnification of the boxed area is ×400. *B*, Epidermal T cells showed a Th0 cytokine profile with high quantities of IFN-γ, IL-5, and IL-13 and relatively lower amounts of IL-4. ○, ◇, and □, unstimulated (u.s.); ●, ◆, and ■, anti-CD2/CD3/CD28 mAb stimulated. *C*, In vitro differentiated $CD4^+$ Th1 and Th2 cells both showed high migration toward IFN-γ- but not TNF-α-stimulated keratinocytes. IFN-γ-stimulated human keratinocytes induced T cell chemotaxis in three experiments with three different T cell sources. SD of duplicate cultures is shown.

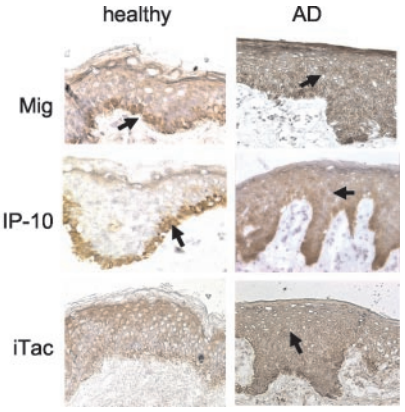

FIGURE 6. Increased IP-10, Mig, and iTac expression in AD skin. Representative images from two healthy donors and eight different AD patients are shown. ×200. Arrows indicate IP-10 and Mig expression on basal layer keratinocytes in healthy epidermis and enhanced Mig, IP-10, and iTac expression on suprabasal keratinocytes in AD.

was expressed both in basal keratinocytes and in suprabasal keratinocytes in normal and AD skin. The expression of IP-10, Mig, and iTac was mainly focused to epidermis. All three chemokines were not expressed in dermis, demonstrating a chemotaxis gradient from dermis to epidermis.

Discussion

Multiple molecules, including families of adhesion molecules and chemokines, provide signals for the dynamic trafficking of T cells into inflammatory tissues. Transendothelial migration and influx into skin represent the first phase leading to dermal perivascular infiltration by T cells in AD. Interestingly, a second step of chemotaxis, as demonstrated in this study, takes place in the migration of T cells closer to and into the epidermis where they augment T cell-mediated effector functions.

Eczematous skin lesions with distinct etiology are associated with T cell infiltration in the dermis leading to an interaction between T cells and keratinocytes and a marked keratinocyte pathology (31). Several studies have demonstrated that IFN-γ is one of the most active cytokines during T cell-keratinocyte interaction (32, 33). IFN-γ-producing T-cells are present in chronic AD lesions (21). Keratinocyte activation-induced cell death by IFN-γ in the activation phase of keratinocytes might strongly contribute to inflammation in AD. Relatively low concentrations of IFN-γ can induce keratinocyte apoptosis and spongioform morphology as an essential mechanism in the pathogenesis of eczematous dermatitis (25, 30). ICAM-1, which is crucial for T cell retention in the epidermis, is expressed on keratinocytes after exposure to IFN-γ (34, 35). IFN-γ up-regulates MHC class I molecules and Fas and induces de novo synthesis of MHC class II molecules on keratinocytes (36, 37). IFN-γ also induces the expression of several cytokines such as IL-1α, IL-1 receptor agonist, TNF-α and GM-CSF (37, 38). TNF-α and IL-17 are able to enhance the efficiency of IFN-γ in activating keratinocytes (35, 39). In addition, IFN-γ was demonstrated to induce squamous differentiation in epidermal keratinocytes. Apoptosis induced by IFN-γ in the present study might be the consequence of terminal differentiation and growth arrest (40). Moreover, whether suppressive cytokines such as IL-10 may inhibit IFN-γ secretion inside the skin during the development of eczema lesions and during the healing process remains to be elucidated (28).

The present study demonstrates that Mig, iTac, and IP-10 mRNAs are expressed in higher amounts after exposure of keratinocytes to IFN-γ. This leads to the migration of T cells from the dermis to the epidermis and represents a second step of chemotaxis after the recruitment of T cells from blood. Arrest and activation of leukocyte integrins via locally expressed chemokines are important steps for the transmigration of T cells through the vascular barrier. Cutaneous lymphocyte-associated Ag, CD45RO, LFA-1 and different chemokine receptors are expressed on the surface of T cells. Beside ICAM-1, surface molecules like CD34, VCAM-1, and E-selectin are expressed on endothelial cells and play an important role in diapedesis together with the receptors on T cells (10). In allergic contact dermatitis, T lymphocytes transmigrate into the dermis driven by an early chemokine gradient. Chemokines, such as RANTES, MCP-1, thymus, and activation-regulated chemokine (CCL17), pulmonary and activation-regulated chemokine (CCL18), and macrophage-derived chemokine (CCL22) are reported to be involved in this process (41). In contrast to chemokines and adhesion molecules in T cell transendothelial migration, the chemokines IP-10, Mig, and iTac are expressed on late stages of contact dermatitis (41). In addition, IP-10 is up-regulated in the epidermis in inflammatory skin diseases like psoriasis or allergic contact dermatitis (42, 43).

CXCR3, the specific receptor for these chemokines, is expressed on T cells, preferentially on Th1 cells (44–46). We detected the

expression of CXCR3 on in vitro differentiated $CD4^+$ Th1 and Th2 cells with Th1 predominance as well as on T cells isolated from epidermis. In addition, epidermal T cells rather showed a Th0 cytokine profile with increased IFN-γ, IL-5, and IL-13 production. After migration from dermis to epidermis, IFN-γ released from epidermal T cells may further induce the expression of more IP-10, Mig, and iTac on keratinocytes and further induce keratinocyte apoptosis. Epidermal T cells also expressed CXCR4, CXCR5, and CCR7, which bind to homeostatic chemokines (4). Interestingly, CCR7 as a central memory T cell chemokine receptor was expressed on epidermal effector T cells (47). The reasons for the expression of these chemokine receptors in epidermal T cells remain to be elucidated. Beside the function of lymphocytes as immunological effector cells, they also produce growth factors such as basic fibroblast growth factor and leukocyte-derived growth factor (48–50). Their later appearance in the eczematous lesions may additionally favor tissue formation and remodeling of the tissue components. Accordingly, Mig and IP-10 are expressed in a later stage of wound healing and are essential for the persistence of lymphocyte recruitment (50). These studies on chemokines and their receptors demonstrate a complex system of different chemokines and chemokine receptors inside the skin during different migration stages of T cells.

The present study demonstrates an active interaction between effector T cells and target keratinocytes in the AD skin. Apparently, during the activation phase of T cell- and IFN-γ-mediated activation-induced keratinocyte death, epidermal keratinocytes release chemokines that attract T cells from dermis to closer to and into the epidermis. The second step of chemotaxis of T cells from the dermis to the epidermis may play an essential role in the augmentation of keratinocyte apoptosis, enhanced infiltration of T cells into the dermis, remodeling of the skin, and chronicity of AD lesions and disease.

Acknowledgments

We thank Andreas Karatsaidis for technical help in immunohistochemistry and Prof. Dr. N. E. Fusenig (Heidelberg, Germany) for providing HaCaT cells.

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6.4 Statement of contribution to publications

For the publication titled “Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells” (J Exp Med., 2004. 199: 1567-1575), I have performed all of the immunocytochemistry, as depicted in figure 5 B. Furthermore, Mübeccel Akdis and I cooperated in the isolation, culture and characterisation of the IL-4-, IL-10- and IFN-γ-secreting cells used for figures 1, 2, 4 and 6, as these were also used for parts of the publication “Absence of T regulatory cell expression and function in atopic dermatitis skin”.

For the publication “A second step of chemotaxis after transendothelial migration: keratinocytes undergoing apoptosis release IFN-γ-inducible protein 10, monokine induced by IFN-γ, and IFN-γ-inducible α-chemoattractant for T cell chemotaxis toward epidermis in atopic dermatitis” (J Immunol., 2003. 171: 1078-1084), I performed several of the experiments with HaCaT keratinocytes that confirmed the results with primary keratinocytes depicted in figure 1. Sven Klunker and I further cooperated in the immunocytochemistry and immunohistochemistry used for figures 3 and 6, respectively.

For the first author publication “Absence of T regulatory cell expression and function in atopic dermatitis skin” (J allergy Clin Immunol., 2006. 117: 176-183), I have performed the experiments used for all figures, apart from those depicted in figure 3A and figure 4.

7. Discussion

Allergic diseases are controlled, mediated and in some cases executed by T cells. The activation, differentiation and interaction of T cells therefore form pivotal points in the understanding of the immunological mechanisms of allergy. This understanding can form the basis for a rational approach to the prevention and treatment of allergic disease.

Traditionally, allergen-SIT was aimed at suppressing the allergen-specific activation of Th2 cells by inducing a shift towards a Th1 response. This idea was based on the hygiene hypothesis, which suggests that allergic disease results from a failure of the immune response to make the normal shift from a Th2-dominated to a predominantly Th1 response within the first 40 days of life, due to a lower exposure to microbial antigens. However, the simultaneous rise of Th1-related disease challenges this theory. We showed that allergy indeed does not result from an aberrant Th2/Th1 balance, but that it is rather the Th2 to Tr1 ratio that decides the outcome of an immune response to allergen (see figure 3). This implicates that active suppression forms an essential mechanism in inducing specific immunological unresponsiveness. The immunological mechanisms that control the shift in dominating allergen-specific subset *in vivo* remain unclear. In contrast to what was previously thought, the cytokine profile of memory T helper cells, and therefore the distinction between a Th1 or Th2 phenotype, may not be consitent, but could very well be dependent on external factors and thus show flexibility [143, 144]. In addition, the Tr1 cells in our study did not appear to be anergic and could expand *in vivo*, as they proliferated under the influence of IL-2, IL-4, IL-7 and IL-15.

We showed, by specifically blocking receptors with mAb or decoy receptor, that the suppressive activity of Tr1 cells depends highly on the suppressive cytokines IL-10 and, to a slightly lower extend, TGF-β. These cytokines have been demonstrated to exert their suppressive effect on the induction of an immune response in several ways. First, both cytokines have

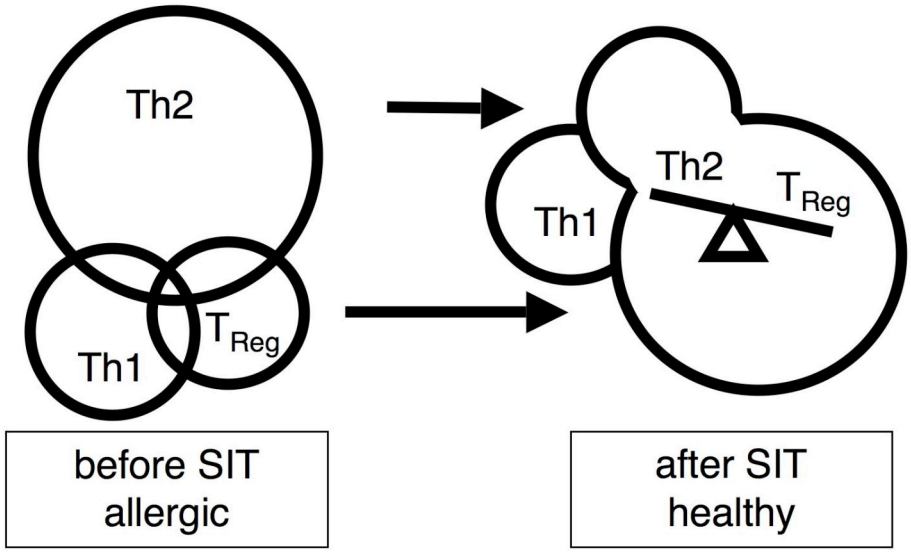

Figure 3. Differences between the healthy and allergic T cell response. A healthy immune response to allergens may not rely on the balance between Th2 and Th1 cells, but rather on the balance between Th2 and Treg cells. The major immunological change after successful allergen-SIT is characterised by a reduced Th2 response and increased numbers of Treg cells. Also, in healthy individuals the majority of allergen-specific T cells is of the Tr1 phenotype, while allergic individuals show a predominantly Th2 response. Adapted from Verhagen J *et al.*, Expert Opin. Biol. Ther. 2005; 5:537-44.

been shown to inhibit the full maturation of DC [145, 146]. Immature DC do not express the co-stimulatory ligands and cytokines needed to successfully initiate a T cell response. Second, IL-10, as previously shown by our group, can inhibit T cell activation by intervening in the interaction of PI3K with the intracellular domain of the co-stimulatory receptor CD28 [147]. This inhibition of the CD28 signalling pathway by IL-10 was recently shown by our group to be mediated by the tyrosine phosphatase SHP-1 (Taylor A *et al.,* manuscript submitted). Furthermore, other co-stimulatory receptors, such as inducible costimulator (ICOS) and CD2, were shown to be subject to IL-10-induced inhibition in a similar

fashion. Interestingly, TGF-β has comparable inhibitory effects on T cell co-stimulation, although a different phosphatase than SHP-1 might be involved (Verhagen J *et al.*, unpublished observation).

Apart from the important role of cytokines in the regulation of the allergen-specific immune response by Tr1 cells, there is also a cell contact-dependent mechanism that plays a role. We demonstrated that blocking CTLA-4 or PD-1 on IL-10-secreting Tr1 cells reduced the suppressive capacity of these cells. The previous observation that the blockade of CTLA-4 is associated with decreased levels of TGF-β [142] could help to explain these results.

A failure in the suppression of T cell activation by innocuous antigen can initiate allergic disease. The pathology of atopic dermatitis results from the migration and differentiation of activated T cells in the dermis of the skin. Once inside the dermis, these T cells differentiate and show a Th1/Th0 cytokine profile with high IFN-γ, IL-5 and IL-13 production. It should be noted here that the receptor affinity and activation thresholds for the Th1 cytokine IFN-γ and the Th2 cytokines IL-4, IL-5 and IL-13 differ significantly. While as little as 1 ng/ml of IFN-γ suffices to induce keratinocyte apoptosis, 50 ng/ml of IL-4 or IL-13 are required to induce IgE production by B cells and the same amount of IL-5 is needed to prolong eosinophil lifespan *in vitro* [9, 148]. The IFN-γ-induced apoptosis of keratinocytes, which might be the result of terminal differentiation and growth arrest, and the subsequent spongiosis formation in the epidermis, are characteristic for atopic dermatitis.

As we demonstrated, a second step of chemotaxis takes place during the migration of T cells closer to and into the epidermis, where they exert their effector functions. We showed that activated keratinocytes actively aggravate their own situation by secreting chemokines that enhance this further migration of T cells. IFN-γ induces the upregulation of the expression of mRNA for the chemokines Mig (CXCL9), iTac (CXCL10) and IP-10 (CXCL11). All three chemokines bind to the receptor CXCR3, which is mostly expressed on Th1 cells and $CD45RO^+$ cells, but also on some Th2 cells. The second step of chemotaxis

may thus play an essential role in the remodelling of the skin and the chronicity of inflammation.

Our group and others, have shown the importance of the interaction between Treg cells and effector T cells in the control of inflammation. The central role of various subsets of Treg cells in the inhibition of antigen-specific T cell activation has been shown in models of allergy, autoimmunity, transplantation, parasitic infection and cancer. Also, several studies have shown a higher number of Treg cells in the blood of healthy individuals compared to those with hyper-immune disease. However, not much is known about the presence and possible role of Treg cells in peripheral tissues, such as the skin, gut or lungs. As already mentioned, IFN-γ induces keratinocytes to secrete the chemokines Mig, IP-10, and iTac, which all share the predominantly Th1-associated chemokine receptor CXCR3 [149], as well as TARC (CCL17) and MDC (CCL22), which bind the Th2-associated receptor CCR4 [150]. Both Tr1 cells and $CD4^+CD25^+$ Treg cells express a vast repertoire of chemokine receptors, including CXCR3 and CCR4, thereby theoretically facilitating their migration into the inflamed skin [151, 152]. Furthermore, the numbers of $CD4^+CD25^+$ T cells and $CLA^+CD4^+CD25^+$ T cells have been demonstrated to be elevated in peripheral blood of AD patients compared to healthy controls or asthma patients [153, 154]. We therefore investigated the presence of $CD4^+CD25^+$ cells *in situ* in the dermis and epidermis of AD and various other skin conditions and found that these cells did not express the transcription factor FoxP3, indicating that they were activated T cells and not Treg cells. Of notice here is that functional mutations in FoxP3 have previously been shown to lead to the rare IPEX syndrome in humans. This disease, also known as X-linked autoimmunity–allergic dysregulation (XLAAD) syndrome, is commonly associated with atopic dermatitis and hyper IgE responses [30]. This suggests that the absence of FoxP3 expression in AD skin could possibly provide a link with the pathogenesis of this disease. In contrast to $CD4^+CD25^+FoxP3^+$ Treg cells, we found large numbers of IL-10- and TGF-β-secreting Tr1 cells in AD skin.

The analysis of T cells isolated from the skin of AD patients showed that these cells highly effectively induce keratinocyte apoptosis, despite the presence of a substantial percentage of IL-10-secreting Tr1 cells. A defect in the sensitivity to regulation has previously been suggested as a determinant in the ongoing effector functions of AD skin T cells. Superantigens, present in the skin of more than 90% of AD patients, can induce strong proliferation of $CD4^+$ and $CD8^+$ T cells in the skin by non-specific crosslinking of MHC-II on antigen-presenting cells or activated KC and the V_β region of the TCR [155]. Strong binding of superantigens to the TCR in conjunction with CD28 co-stimulation [156] was shown to render T cells insensitive to suppression by $CD4^+CD25^+$ Treg cells [157, 158] and IL-10 [159]. Superantigens also abrogate immune suppression by corticosteroids [160], which, recent evidence suggests, operate via the induction of Treg cells [161].

Subsequent *in vitro* experiments with cells that were isolated from peripheral blood and had not been exposed to superantigens, confirmed that Tr1 and $CD4^+CD25^+$ Treg cells are able to suppress allergen-specific activation of effector T cells. However, neither of these Treg cell subsets was able to suppress keratinocyte apoptosis induced by pre-activated Th1 cells. Further assays based solely on cytokines and keratinocytes, as well as culture experiments with artificially generated, fully differentiated skin segments confirmed that Treg cells are not able to suppress the effector function of pre-activated T cells.

In perspective, four distinct stages play an important role in allergic inflammation of the skin (figure 4). 1, the activation of T cells by APC through the presentation of a wide range of antigens (e.g. aeroantigens, food antigens or autoantigens) on their surface or by the encounter of superantigen. 2, organ-selective homing, whereby cells are influenced by a network of chemokines and are drawn towards and into the skin. 3, the prolonged survival of T cells within the inflamed skin, due to contact with the ECM and the encounter of survival cytokines, coinciding with a second step of reactivation, mainly by superantigens. 4, the effector roles of T cells, which result in the induction of hyper IgE production, the activation of

eosinophils, the induction of keratinocyte apoptosis and the development of spongiosis, all of which are important factors in the pathogenesis of AD.

The studies in this thesis demonstrate that Treg cells of either Tr1 or $CD4^+CD25^+$ phenotype can suppress allergen-specific activation of effector T cells (stage 1 and, if not stimulated by superantigen, stage 3) and therefore play an important protective role in the development of allergic disease. Furthermore, the upregulation of the number of Treg cells by allergen-SIT can lead to the induction of an immunological state whereby a response to allergen exposure is no longer initiated. However, in established disease such as AD, where T cells have been pre-activated, and have migrated to the tissues under the influence of a specific homing receptor or a chemokine network, neither Treg cells, nor their suppressive cytokines IL-10 and TGF-β, are able to directly suppress the destructive effector functions of activated T cells (stage 4).

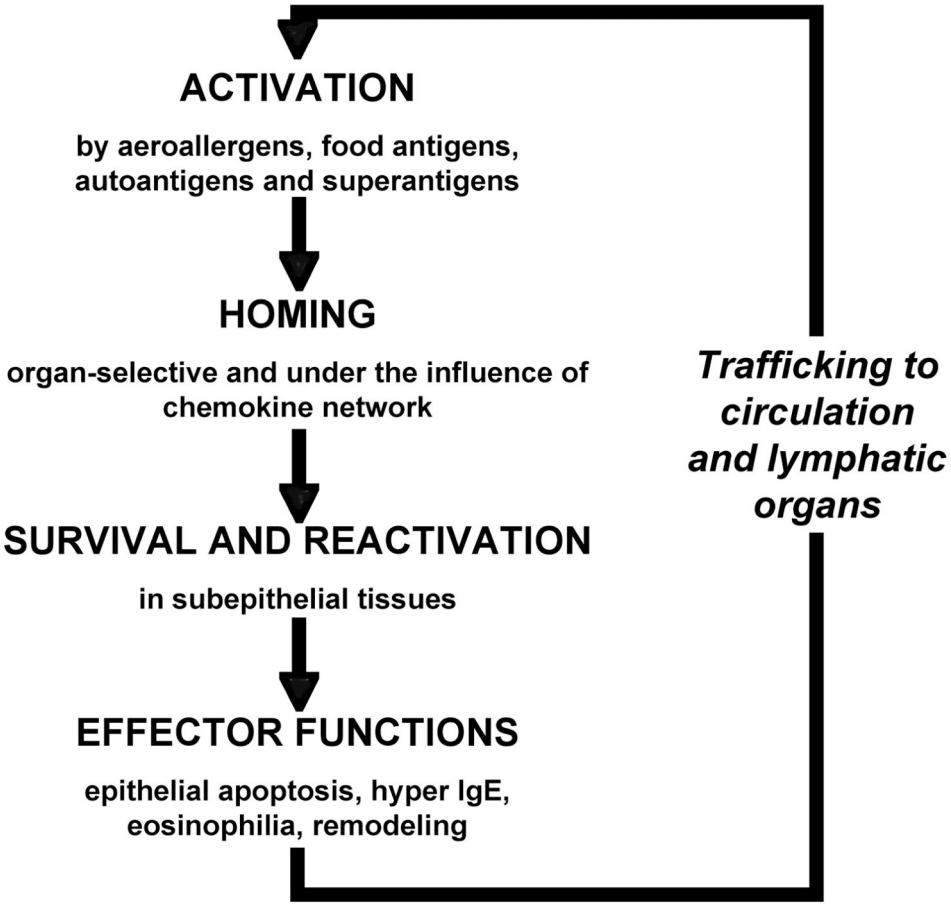

Figure 4. The four sequential processes characterising allergic inflammation.

(1) A wide range of known antigens and yet unidentified factors can activate T cells. (2) Subsequently, these T cells undergo organ-selective homing under the influence of organ-related chemokine networks. (3) T cells within subepithelial tissues show increased survival due to contact with the extracellular matrix and locally produced cytokines, while repeatedly being stimulated. (4) These activated T cells meanwhile secrete their specific cytokines, leading to apoptosis, hyper IgE and eosinophilia. Adapted from Taylor A *et al.*, Int. Arch. Allergy Imunol. 2004; 135:73-82

8. Curriculum Vitae

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Type of certification: M.Sc.

PhD studies:
Matriculated at Zürich University since: summer semester 2002
Employed at the Swiss Institute of Allergy and Asthma Research (SIAF), Davos since: April 2002

Examinations and courses during PhD:

Oral examination in Biochemistry with Prof. Dr. H.R. Bosshard, Zürich University, June 2004

Written examinations following Immunology lectures at SIAF, moderated by Prof. Dr. K. Blaser, PhD, Prof. Dr. C.A. Akdis, MD, Prof. Dr. R. Crameri, PhD, Dr. C. Schmidt-Weber, PhD and PD Dr. M. Akdis, MD, PhD. winter semester 2004/5 and summer semester 2005

Presentations given in SIAF

Progress report	Journal club
10 July 2002	26 June 2002
25 June 2003	27 November 2002
26 November 2003	7 May 2003
19 May 2004	12 November 2003
17 November 2004	30 June 2004
17 May 2005	5 April 2005
27 September 2005	30 August 2005

Congress attendance

XVth meeting of the Swiss immunology PhD students, March, 2003. Schloss Wolfsberg, Ermatingen, Switzerland
Poster presentation

Annual meeting of the SCS (Swiss cytometry society), November 27-28, 2003, Lausanne, Switzerland
No presentation

XVIth meeting of the Swiss immunology PhD students, March 31-April 2, 2004. Schloss Wolfsberg, Ermatingen, Switzerland
Oral presentation

Annual meeting of the SSAI-SGAI (Swiss Society for Allergology and Immunology), April 15-17, 2004, Geneva, Switzerland.
Poster presentation

XXIIIrd EAACI (European Academy of Allergy and Clinical Immunology) congress, June 12-16, 2004, Amsterdam, The Netherlands.
Poster presentation, awarded with JMA (Junior Member Association) Poster Prize.
Oral presentation

20. Fortbildungskongress "Fortschritte der allergologie, immunologie und dermatologie", Davos, Switzerland, September 1-4, 2004
Poster presentation

3rd EAACI Davos meeting, February 3-6, 2005. Davos, Switzerland
Poster presentation

XVIIth meeting of the Swiss immunology PhD students, March 30-April 1, 2005. Schloss Wolfsberg, Ermatingen, Switzerland
Oral presentation

Publications

Articles in peer-reviewed journals

Klunker, S., Trautmann, A., Akdis, M., Verhagen, J., Schmid-Grendelmeier, P., Blaser, K. and Akdis, C.A. (2003) A second step of chemotaxis after transendothelial migration: keratinocytes undergoing apoptosis release IFN-gamma-inducible protein 10, monokine induced by IFN-gamma, and IFN-gamma-inducible alpha-chemoattractant for T cell chemotaxis toward epidermis in atopic dermatitis. J Immunol, 171, 1078-84.

Akdis, M., Verhagen, J., Taylor, A., Karamloo, F., Karagiannidis, C., Crameri, R., Thunberg, S., Deniz, G., Valenta, R., Fiebig, H., Kegel, C., Disch, R., Schmidt-Weber, C.B., Blaser, K. and Akdis, C.A. (2004) Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med, 199, 1567-75.

Taylor, A., Verhagen, J., Akdis, C.A. and Akdis, M. (2004) T regulatory cells in allergy and health: a question of allergen specificity and balance. Int Arch Allergy Immunol, 135, 73-82.

Verhagen, J., Taylor, A., Akdis, C.A. and Akdis, M. (2005) Advances in allergen-specific immunotherapy. Expert Opin Biol Ther, 5, 537-44.

Verhagen, J., Taylor, A., Akdis, M. and Akdis, C.A. (2005) Targets in allergy-directed immunotherapy. Expert Opin Ther Targets, 9, 217-24.

Taylor, A., Verhagen, J., Akdis, C. A. and Akdis, M. (2005) T regulatory cells and allergy. Microbes Infect, 7, 1049-55.

Verhagen, J., Taylor, A., Akdis, M. and Akdis, C.A. (2005) T regulatory cell in allergen-specific immunotherapy. Int Rev Immunol, 24, 533-48.

Verhagen, J., Akdis, M., Traidl-Hoffmann, C., Schmid-Grendelmeier, P., Hijnen, D., Knol, E.F., Behrendt, H., Blaser, K. and Akdis, C.A. (2006) Absence of T-regulatory cell expression and function in atopic dermatitis skin. J Allergy Clin Immunol, 117, 176-83.

Unreviewed articles

Akdis, M., Verhagen, J., Blaser, K. and Akdis, C.A. (2005) Role of T cells in atopic eczema. Handbook of atopic eczema 2nd edition, eds. J. Ring, B. Przybilla, T. Ruzicka. Springer-Verlag, Berlin, 323-31.

Taylor, A., Verhagen, J., Akdis, C.A. and Akdis, M. (2004) T regulatory cells: allergen specificity and balance. T J Immunol, 9, 1-14.

Awards

JMA (Junior Member Association) Poster Prize.
XXIIIrd EAACI (European Academy of Allergy and Clinical Immunology) congress, June 12-16, 2004, Amsterdam, The Netherlands.

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